The Evolution of the Matrix Genes of Human Influenza A and Relationships to Functional Properties

Thesis

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The Evolution of the Matrix Genes of Human Influenza A and Relationships to Functional Properties

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Declaration

I have not submitted any portion of the work referred to in this thesis in support of an application for another degree or qualification of this or any other university or institute of learning.

The work carried out in this thesis was performed by myself unless otherwise indicated in the text.

Part of this work has already been published or is currently in press:

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Abstract

Amantadine and rimantadine are anti-influenza A drugs that target the M2 protein, located within the viral envelope. Over the last century, the circulating human influenza A viruses in the UK have included H1N1, H2N2 and H3N2 antigenic subtypes. The main aim of this study was to analyse the national archive of influenza A viruses for amantadine susceptibility as there is limited data on natural resistance over the entire period of circulation of influenza A viruses. Over 2300 influenza A viruses were screened from a period representing all influenza seasons and antigenic variants over the last 40 years in the UK. Phenotypic and genotypic analyses determined the frequency of amantadine-resistance. Enzyme-linked immunoassay and plaque reduction assay determined resistance to be 2.4% and 2.1% for H3N2 and H1N1 viruses, respectively. No evidence of amantadine-resistance was found in the limited number of H2N2 viruses screened. Amino acid mutations within the M2 protein transmembrane domain were found in 22/48 of resistant viruses. Serine to asparagine substitutions at amino acid position 31 were most frequent, conferring resistance in 9/22 viruses. A large group of phenotypically resistant viruses did not contain any M2 transmembrane mutations; no other mutations within the matrix genes could be attributed to the resistance. The occurrence of resistance over time was sporadic and often occurred in clusters. Some of the clusters were traced to outbreaks of influenza where amantadine had been used to limit the spread of disease. The emergence of natural resistance within the population was low and did not persist through influenza seasons. Analysis of the M gene sequence indicated that the two matrix gene products, M1 and M2, evolved independently of each other. M2 evolved at a faster rate of nucleotide and amino acid change than M1.

Twelve influenza A H3N2 viruses were isolated from a persistent infection of an immunocompromised host. An amantadine-resistant virus population emerged following a short course of amantadine treatment and was maintained for eighteen months in the absence of drug pressure. Virus isolates were mixtures of amantadine-sensitive and -resistant variants, which varied over time. Mutations within the haemagglutinin (HA) molecule suggested that the viral population had undergone antigenic drift over the study period. There was no detectable immune response within the host, therefore the drift, or evolution of these viruses had occurred without the selection pressure of a competent immune system. Fixed

mutations within antigenically important regions of the HA molecule were found to affect receptor-binding properties of the viruses. The HA molecule had an increased rate of evolution compared to field virus strains that circulated over the same period as the persistent infection. The emergence and progression of amantadine-resistance and viral evolution within the normal and immunocompromised host will be discussed.

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Abbreviations

°C	degrees centigrade
µl	microlitre(s)
aa	amino acid(s)
Ab	antibody
AD	agglutinating dose
bp	base pair(s)
CPHL	Central Public Health Laboratory
cRNA	copy ribonucleic acid
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EIA	enzyme immunoassay
ELISA	enzyme-linked immunoassay
E. coli	*Escherichia coli*
FDA	Food and Drug Administration
HA	haemagglutinin
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
HIV	human immunodeficiency virus
hr	hour
HRPO	horseradish peroxidase
ILI	influenza-like-illness
l	litre
KDa	Kilodaltons
M	matrix
MAb	monoclonal antibody
MDCK	Madin Darby canine kidney
MEM	minimal essential medium
min	minute(s)
ml	millilitre
mM	millimolar / millimole
mRNA	messenger ribonucleic acid
NA	neuraminidase
NI	neuraminidase inhibitor

NICE	National Institute for Clinical Excellence
NP	nucleoprotein
NPA	nasopharengeal aspirate
NS	non-structural protein
nt	nucleotides
OD	optical density
PBS	phosphate buffered saline
PBST	phosphate buffered saline (Tween20)
PCR	polymerase chain reaction
PFU	plaque forming units
Pfu polymerase	*Pyrococcus furiosus* polymerase
PHLS	Public Health Laboratory Service
RCGP	Royal College of General Practitioners
RDE	receptor destroying enzyme
RMK	rhesus monkey kidney
RNA	ribonucleic acid
RNP	ribonucleoprotein
RPM	revolutions per minute
RSV	respiratory syncytial virus
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
sec	second(s)
Taq polymerase	*Thermus aquaticus* polymerase
TBE	tris borate EDTA
TGN	trans Golgi network
TMB	3,3',5'5'-tetramethylbenzidine
UK	United Kingdom
US	United States of America
VTM	virus transport medium
vRNA	viral ribonucleic acid
WHO	World Health Organisation

Amino Acid Nomenclature

Full Name	Three-letter code	Single-letter code
Alanine	Ala	A
Arganine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Chapter 1

General Introduction

1.1 The History of Influenza Episodes

The identification of influenza epidemics and pandemics from historical records and anecdotal evidence is fraught with difficulty. Over the past 250 years, at least ten, and perhaps as many as twenty pandemics have swept the globe interspersed with a much larger number of milder, more localised epidemics. Influenza appears to have afflicted human beings since ancient times, perhaps the earliest recorded epidemic was in 412 BC when symptoms and traits of a disease that could have been influenza was recorded by Hippocrates (285). Numerous episodes of influenza were described during the Middle Ages, but it wasn't until the early 15th century in Italy that the term influenza was introduced, describing an epidemic that was attributed to the influence of the stars (177).

The first pandemic with well-defined records occurred in 1580 and was believed to have originated in Asia from where it spread to Africa and Europe. The whole of Europe was affected from North to South in a six-month period, and infection subsequently spread to North America. From 1700 data concerning infiuenza pandemics is more informative and there are several documented episodes occurring throughout that century. The first truly global pandemic to be described was that of 1889. That pandemic was better documented than any other previous episode, since communications and travel were still relatively slow, the spread of infection was monitored without too much confusion (285).

Of all pandemics, none proved to be more of a threat to human survival than the one of 1918-19. Estimates of mortality run from a minimum of 20 million deaths globally, to as many as twice that number (266, 299). Influenza killed more people in a few months than all the armies of the 1914-18 war in five years. The US armed forces were particularly badly hit, about 80 percent (43,000) of the total number of American deaths were accounted for by influenza in 1918 (62). Why this pandemic was so lethal is still not clear, but an important factor was probably a marked increase in the virulence of the virus during the first phases of the pandemic. Certainly, in the pre-antibiotics age, secondary bacterial infections causing pneumonia and other complications accounted for many, if not most, of the deaths. The pandemic has been extensively chronicled and large selections of literature review the events in countries, towns and military camps, and personal experiences (62, 121, 365). Recent work has been undertaken to establish the

genetic sequence of the 1918 virus, in particular to attempt to identify the causes behind its obvious virulence. Sequencing of the major surface glycoproteins has been accomplished but this has not revealed the underlying markers for virulence (297, 298, 354). Current efforts have now been focused on sequencing the major internal genes of the virus (21).

Man has speculated on the cause of influenza for centuries. By the end of the 19th century, when the microbiological concept of infectious disease had taken root, a bacillus was discovered in the throats of many influenza patients that remained a leading suspect as the causative agent of influenza for many years. It was not until the late 1920s that the true viral cause of influenza was established when it was shown that swine influenza could be transmitted with filtered clinical samples (327). Initial studies on swine influenza characterised the disease produced in the animals and established the presence of different strains of the same virus (326). During an epidemic of influenza sweeping London in 1933, Smith *et al.* (1933) isolated influenza virus from the throat washings of patients (335). Washings were filtered and inoculated into a range of test animals with little success until some ferrets used in canine distemper studies were inoculated. The animals developed classic signs of the disease and it was found that the virus could be transmitted to healthy ferrets either by inoculation of infected tissue or by simple contact (335). Smith next performed an experiment using ferrets infected with a swine influenza virus from Shope's earlier studies and found that the animals developed disease that was indistinguishable from that produced by virus of human origin. Shope (1934) confirmed Smiths findings and performed neutralisation studies using both swine and ferret sera (325). Influenza was first grown in cell culture during attempts to cultivate the virus on the chorio-allantoic membrane of developing hens' eggs when virus was grown in minced chick embryo suspended in Tyrode's solution medium (334).

Since the original isolation of human influenza in 1933, pandemics have been accurately identified as a direct result of the isolation and identification of the viruses responsible. It has been agreed that since 1933 there have been three definite pandemic events (1957-58, 1968-70 and 1977-78) (52, 81, 197) and one episode that was not considered a pandemic only because it was not truly global (1946-48) (186). As influenza has the potential to be economically and socially

devastating, it is important to analyse records from the past to gather vital information that might help predict or prevent pandemics of the future.

1.2 The Virus

1.2.1 Taxonomy

Three types of influenza virus (A, B and C) and thogotoviruses comprise the family *Orthomyxoviridae*. The influenza viruses are differentiated into the three types, A, B and C, on the basis of differences in the major internal proteins, the nucleoprotein (NP) and matrix (M1) protein (309). Influenza A viruses have an animal reservoir whereas types B and C are found only in man. Influenza A viruses are further divided into subtypes based on differences in the haemagglutinin (HA) and neuraminidase (NA) genes. There are fifteen subtypes of HA of which only three, H1, H2 and H3 are frequently associated with human infection, and nine subtypes of NA of which two, N1 and N2 have been associated with human infection (382) (Table 1.1). The natural reservoir of influenza A is considered to be aquatic fowl as all influenza subtypes are found in wild birds whereas there is only limited distribution of subtypes in certain mammal species (383).

1.2.2 Particle Structure

Influenza viruses are small (80-120 nm in diameter) pleomorphic particles that are generally spherical but can also be isolated in a filamentous state (302) (Figure 1.1). The surface of the virus is covered with a layer of radial glycoprotein projections; there are two different types of glycoprotein spike, HA and NA (Figure 1.2). A third membrane protein, M2 is present embedded in the virus lipid membrane; the function of this protein is to form an ion channel (282).

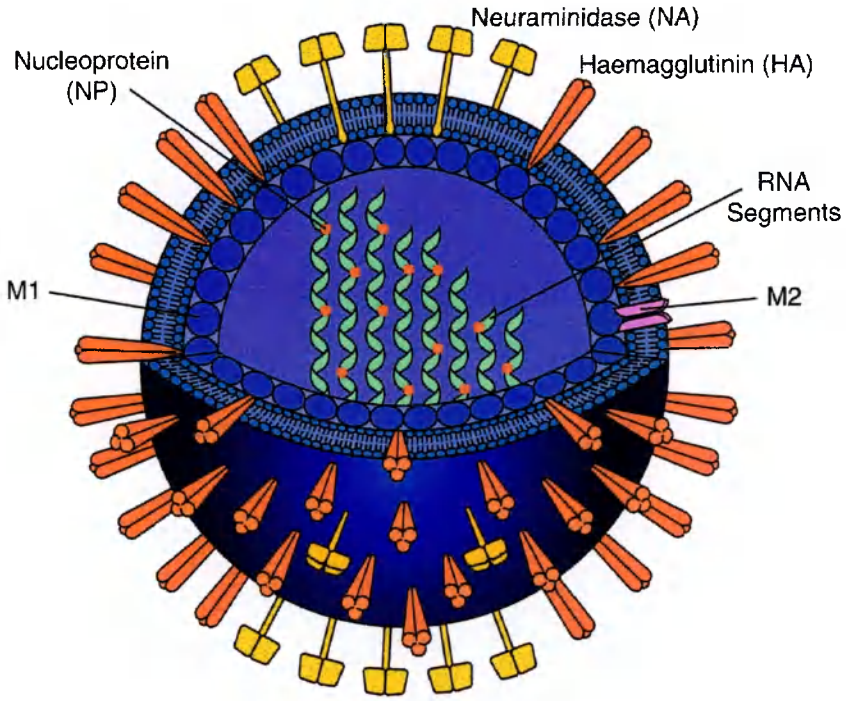
TABLE 1.1 HA and NA subtypes of influenza A viruses isolated from humans, lower animals, and birds

	Virus Found in Species of Origin[a]			
Subtype	**Humans**	**Swine**	**Horses**	**Birds**
HA				
H1	PR/8/34	Sw/Ia/15/30	-[b]	Dk/Alb/35/76[c]
H2	Sing/1/57	-	-	Dk/Ger/1215/73
H3	HK/1/68	Sw/Taiwan/70	Eq/Miami/1/63	Dk/Ukr/1/63
H4	-	-	-	Dk/Cz/56
H5	-	-	-	Tern/S.A./61
H6	-	-	-	Ty/Mass/3740/65
H7	-	-	Eq/Prague/1/56	FPV/Dutch/27
H8	-	-	-	Ty/Ont/6118/68
H9	-	-	-	Ty/Wis/1/66
H10	-	-	-	Ck/Ger/N/49
H11	-	-	-	Dk/Eng/56
H12	-	-	-	Dk/Alb/60/76
H13	-	-	-	Gull/Md/704/77
H14	-	-	-	Dk/Gurjev/263/82
H15	-	-	-	Dk/Aust/341/83
NA				
N1	PR/8/34	Sw/Ia/15/30	-	Ck/Scot/59
N2	Sing/1/57	Sw/Taiwan/70	-	Ty/Mass/3740/65
N3	-	-	-	Tern/S.A./61
N4	-	-	-	Ty/Ont/6118/68
N5	-	-	-	Sh/Austral/1/72
N6	-	-	-	Dk/Cz/56
N7	-	-	Eq/Prague/1/56	FPV/Dutch/27
N8	-	-	Eq/Miami/1/63	Dk/Ykr/1/63
N9	-	-	-	Dk/Mem/546/74

[a] - The reference strains of influenza viruses, or the first isolates from that species are presented. [b] - Not found in this species. [c] - Subtypes are based upon the host of origin, geographic origin, strain number and year of isolation; the subtype is given in parenthesis, e.g. A/Equine/Prague/1/56 (H7N7). The host of the human influenza A strains is excluded from this nomenclature e.g. A/Singapore/1/57 (H2N2). Adapted from (383) and (305).

FIGURE 1.1 Electron micrograph showing a cluster of influenza A virions

FIGURE 1.2 A schematic view of the influenza A virion structure



1.2.3 The Genome

Influenza viruses are single-stranded RNA viruses in the negative sense with a segmented genome. Influenza A and B virus genomes consist of eight segments of RNA and influenza C virus consists of seven. Each segment of the genome is independently encapsidated by the viral nucleoprotein (NP) and associated with a polymerase complex (309). The ribonucleoprotein (RNP) particle consists of viral RNA (vRNA), NP, and the polymerase complex. The polymerase complex is made up of three proteins, PB1, PB2, and PA (383). The RNP particles lie within a shell consisting of M1 protein, which lines the viral lipid membrane. The viral lipid membrane of influenza virus is acquired from the modified plasma membrane of a host cell during the process of budding after infection. Table 1.2 summarises the genome segments of influenza A and their gene products.

TABLE 1.2 Virus specified gene products of influenza A

Segment	Length (nt)	Encoded Polypeptide	Polypeptide Length (aa)	Molecular Weight	Approx. no. Molecules per Virion	Function	Reference
1	2341	PB2	759	85700	30-60	Recognition of & binding to host cell cap structure Transcriptase activity	(187) (194)
2	2341	PB1	757	86500	30-60	Transcriptase activity	(194)
3	2233	PA	716	82400	30-60	Polymerase Function not clear	
4	1778	HA	548	77000	500	Acid sensitive glycoprotein	(366)
		HA1	326	50000		Receptor binding sites	
		HA2	222	27000		Forms stem structure of HA	
5	1565	NP	498	56101	1000	Encapsidates RNA in RNP	
6	1413	NA	454	50087	100	Facilitates virus release Prevents self-aggregation	(135)
7	1027	M1	252	27801	3000	Inhibits vRNA transcription Interacts with RNP	(133) (415)
		M2	97	11010	20-60	Ion channel	(281)
8	890	NS1	230	26815		Binds to vRNA	(232)
		NS2	121	14216		Regulation of M1 function	(378)

1.2.4 Replication cycle

Studies on the replication cycle of influenza viruses have determined the approximate time for an infectious single cycle and each different stage. Following initial infection, detection of viral antigens at the cell surface is possible after approximately 4-5 hours, and budding of progeny virions from cell membranes after 5-6 hours (343). Compared to other viruses this is a relatively short cycle e.g. herpes simplex has a replication cycle of approximately 18 hours (390). The cycle can be categorised into 5 basic stages: 1) attachment and internalisation, 2) uncoating, 3) replication of genetic material, 4) assembly and 5) budding.

HA attaches to sialic acid containing glycoprotein and glycolipid receptors on target cells (84). The virus enters the cell by host cell-mediated endocytosis (235). The acidic environment within the endosome promotes the uncoating and release of the RNP core. This triggers a conformational change of the HA, which promotes fusion of viral and endosomal membranes releasing the contents of the endosome into the cytoplasm (106). Prior to this event, the lowered endosomal pH activates the M2 ion channel that opens, allowing H^+ ions to flow into the virion. This causes an acid-induced dissociation of the matrix protein from the RNP that allows the migration of the RNP through the cytoplasm into the nucleus (39).

In the early stages of virus replication the viral RNP is used as a template by the viral RNA polymerase complex to synthesise two different species of RNA (141). These are an mRNA species with a 5'-cap and poly(A) tail, and a complementary RNA (cRNA) that represents a full length copy of the viral RNA (vRNA) (174). PB2 of the viral polymerase complex recognises and binds to the methylated caps at the 5' termini of host cell mRNA. Endonuclease activity cleaves this short sequence that then acts as a primer for viral mRNA synthesis. The mRNAs initially synthesised are incomplete transcripts with additional short length sequences attached at both 5' and 3' ends. In order for there to be production of genomic RNA, exact complementary template cRNAs are synthesised with no additional sequences (141).

In the influenza replication cycle, there are two expression stages, early and late. The early stage follows primary transcription and predominantly involves the synthesis of the NP and NS genes (322). The synthesis of these proteins reflects

their importance in the regulation of transcription and replication. It is believed that synthesis of the NP and NS triggers a switch from mRNA synthesis to cRNA and vRNA synthesis. During late stages of gene expression, the structural proteins are synthesised and splicing of M and NS genes occurs (139). PB1, PB2, PA and NP contain signals necessary for migration into the nucleus where they assemble into functional complexes and nucleocapsids (199). Nucleocapsids are then exported into the cytoplasm where a complement of genome RNP segments are assembled by a mechanism that is still poorly understood (139).

The structural proteins, HA, NA and M2 are synthesised in the endoplasmic reticulum and inserted into its membrane. During the transport of the structural proteins through the trans Golgi network (TGN) to the plasma membrane, the proteins mature and modify into their multimeric states (139). The proteins are incorporated into the cell plasma membrane and M1 associates with the membrane. It is thought that M1 forms the basis for interactions whereby vRNP associate with M1, assemble and bud out of the infected cell. The basis of these interactions is poorly understood.

1.2.5 Polymerase Complex (PB1, PB2, PA)

The influenza virus polymerase molecule exists as a complex formed by the PB1, PB2 and PA proteins (73). During viral replication, the complex is responsible for the synthesis of three classes of RNA molecules; 1) mRNA species that are capped and polyadenylated, 2) vRNA and 3) cRNA that serves as a template for vRNA synthesis (195). Each subunit of the polymerase complex has a unique function. The role of the PB1 subunit in the viral replication cycle is to aid elongation of the mRNA chain. Located within the PB1 protein is a series of motifs that are typical of an RNA-dependent RNA polymerase (284). The PB2 subunit binds to cap structures and is thought to be responsible for the initiation of transcription (28, 35). Studies have shown that replication of vRNA requires the PB2 subunit of the polymerase complex indicating the importance of this protein (279). The function of the PA subunit is not fully understood but it is thought to be involved in the synthesis of vRNA during the virus replication cycle.

1.2.6 Non-Structural (NS1, NS2)

Segment eight of the influenza A virus genome encodes two overlapping polypeptides, NS1 and NS2 (201). NS1 is an RNA-binding protein and the only true non-structural influenza protein, only found in infected host cells. NS1 has been shown to have several important functions including; 1) the inhibition of host mRNA polyadenylation, which may contribute to virus-induced shutoff of host protein synthesis (257), 2) the inhibition of nuclear export of mRNA (289) and 3) preventing the host interferon-mediated antiviral responses (104).

Current studies have investigated the role of NS1 in controlling viral virulence; transfectant viruses lacking the NS1 gene (delNS1) have reduced growth in tissue culture and eggs when compared to wild-type viruses (104). The inhibition of virus growth is linked with the interferon responses in these systems. When delNS1 virus is inoculated into interferon-lacking systems, the virus demonstrates wild-type growth, but in interferon-competent systems the delNS1 viruses have altered growth properties (104). Therefore, it appears that NS1 plays an important role in inhibiting the interferon-mediated antiviral responses of the host. This work has created a novel approach to vaccine production. Viruses with altered NS1 proteins have been shown to be highly attenuated in mice, but provide good protection when the host is challenged with wild-type virus (353).

The NS2 protein is synthesised after splicing of NS1 mRNA (201). Although originally considered a non-structural protein, NS2 has been shown to be associated with purified influenza particles (403). The NS2 protein is thought to contain the specific nuclear export signal required to mediate vRNA export from the infected host cell nucleus and thus, is also referred to as the nuclear export protein (265). It is thought that the M1 protein plays an important role in nuclear export of vRNA, studies have shown a specific molecular association between NS2 and M1 (403).

1.2.7 Nucleoprotein (NP)

The influenza A virus genome is composed of ribonucleoprotein complexes consisting of the three polymerase molecules, and the nucleoprotein (NP). The NP

is a major structural protein and has been implicated as a major factor in determining host specificity of influenza A H3N2 viruses (318). NP has multiple functions in the replication cycle of influenza viruses. NP encapsidates the naked RNA, it has been estimated that approximately one protein molecule interacts with 20 RNA nucleotides (47). It is thought that multiple regions of the NP are involved in RNA binding, several sequences within the C-terminal two-thirds of the protein are in direct contact with the RNA (89).

NP is also a critical factor in the influenza replication cycle, it has been implicated in switching viral RNA synthesis from transcription to replication. The processes involved in mRNA transcription and vRNA replication are different, therefore the switch is essential for vRNA replication to occur. Although the exact role of NP in this process is not clear, there is evidence to suggest that the NP interacts with the influenza virus polymerase complex (27). It is suggested that NP binding to the PB2 unit might affect either the 5'-cap recognition or cap cleavage function of PB2 resulting in cap-independent initiation of RNA synthesis (27).

1.2.8 Haemagglutinin (HA)

The HA molecule of influenza is an integral membrane glycoprotein and is encoded by RNA segment 4 (339, 383) (Table 1.2). HA is synthesised as a precursor, HA0; the mature HA consists of a 220 kDa trimer of identical subunits, each of which consists of two disulphide-linked glycopeptides, HA1 and HA2 (192). HA1 primarily forms a membrane-distal globular head, and HA2 a central helix-rich stem structure (396).

Maturation and post-translational processing of HA occurs, consisting of three main events. There is cleavage of the amino terminal hydrophobic signal sequence of 14-18 amino acids, addition of N-linked carbohydrate side chains, and covalent attachment of palmitic acid to cysteine residues near the HA carboxy terminus (339, 383). Cleavage of HA0 is considered to be the final processing step. It is cleaved into HA1 and HA2, linked by a single disulphide bond between residues 141 and 1372. This proteolytic processing of HA0 activates the membrane fusion potential of HA1 and therefore it is essential for virus infectivity (192, 396).

Influenza HA mediates the binding of the virus to target cell surfaces during the infection cycle. It is known that cell surface glycoproteins and glycolipids contain sialic acids that are receptors for influenza virus (236). Initial X-ray structure analysis of the HA molecule revealed that the receptor binding sites for sialic acid are located at the distal tip of each subunit of the HA trimer (396). Binding sites consisted of pockets of conserved residues whilst other residues surrounding the conserved pocket varied, especially when undergoing antigenic drift (396). Binding to sialic acids can differ between different strains of influenza virus. For example, human influenza A strains bind preferentially to terminal sialic acids attached with α-(2,6) linkages while avian strains favour binding to α-(2,3) linked sialic acid, it is thought that there are differences in the way that receptors with these linkages bind to HA (57).

Following attachment to host cells and internalisation of virus particles by host cell-mediated endocytosis, viral nucleic acid is released into the infected cell by a process involving the fusion of host and viral membranes (406, 44). Cellular proton pumps progressively acidify the endosomal compartments, as conditions within the endosome approach pH5, the HA undergoes an irreversible conformational change (366). One consequence of this change is that the amino terminal of HA2, normally buried in the interior of the native HA trimer, becomes exposed. This hydrophobic domain of HA2 is commonly referred to as the 'fusion peptide' because it is thought to have an important role in fusion possibly through interactions with target membranes (339). The HA stalk becomes inverted which brings the viral and target membranes closer together, and with the fusion peptide relocated in its correct position, membrane fusion occurs (235).

An important feature of HA is that it functions as a primary target for neutralising antibodies within the host. However, the RNA polymerase of influenza is extremely error-prone which produces a very high mutation frequency (338). Consequently, the HA is subject to a very high rate of mutation, estimated to be about 5.3×10^{-3} nucleotide substitutions per site per year (96). Influenza viruses are able to evade the immune system through selecting viruses with mutations in the HA molecule which result in either failed or reduced antibody binding. Mutations occur in the globular domain of HA1 around the conserved receptor-binding sites (339, 393).

1.2.9 Neuraminidase (NA)

The NA molecule of influenza is a tetrameric membrane glycoprotein (247). It has a molecular mass of 240 kDa and electron microscopy studies of negatively stained material reveal that the protein has a mushroom-shaped morphology (400). The protein is membrane-anchored by a hydrophobic stalk peptide, and it has been shown that this anchor is located near the N-terminus of the NA molecule (53). Attached to the NA stalk is a globular head that contains the active enzyme site and the antigenic sites of the molecule (54).

Sequence comparisons of the catalytic site of the NA molecule have revealed that the structure is highly conserved (54). It has been shown that within the globular head regions, any two NA subtypes are of the order of 45 percent identical and sequence variation within subtypes is very low (53). The discovery that the catalytic site of the NA molecule shared sequence homology between all influenza viruses led to the realisation that inhibitors directed against this enzyme may have provided an effective target antiviral target influenza (374). Several NA inhibitors, e.g. RWJ-270201 are currently undergoing clinical trials while others e.g. zanamivir and oseltamivir have been licensed for clinical use (154, 228, 330).

The NA enzyme catalyses the cleavage of both α-(2,3) and α-(2,6) linkages between sialic acid residues and glycoconjugates (398). Sialic acid is an integral part of the influenza virus receptor and therefore the virus, through the activity of NA, destroys its own receptor (158). By destroying these HA receptors NA allows progeny virus particles to bud from infected cell surfaces and subsequently be released (222). When a virus is cultured in the presence of a neuraminidase enzyme inhibitor e.g. FANA, the result is that the virus is restricted to a single replication cycle (271). This is due to the fact that progeny virus are immobilised at the cell surface, evidence of which can be observed by electron microscopy when virions can be seen in clusters and chains at the surface of infected cells (271).

Another function of NA is that it facilitates the passage of virus through protective mucin covering target cells of the respiratory tract by desialyation of the sialic acid rich mucin (191). It is also thought that NA prevents aggregation by HA of freshly synthesised viral glycoproteins via sialyated carbohydrates (368).

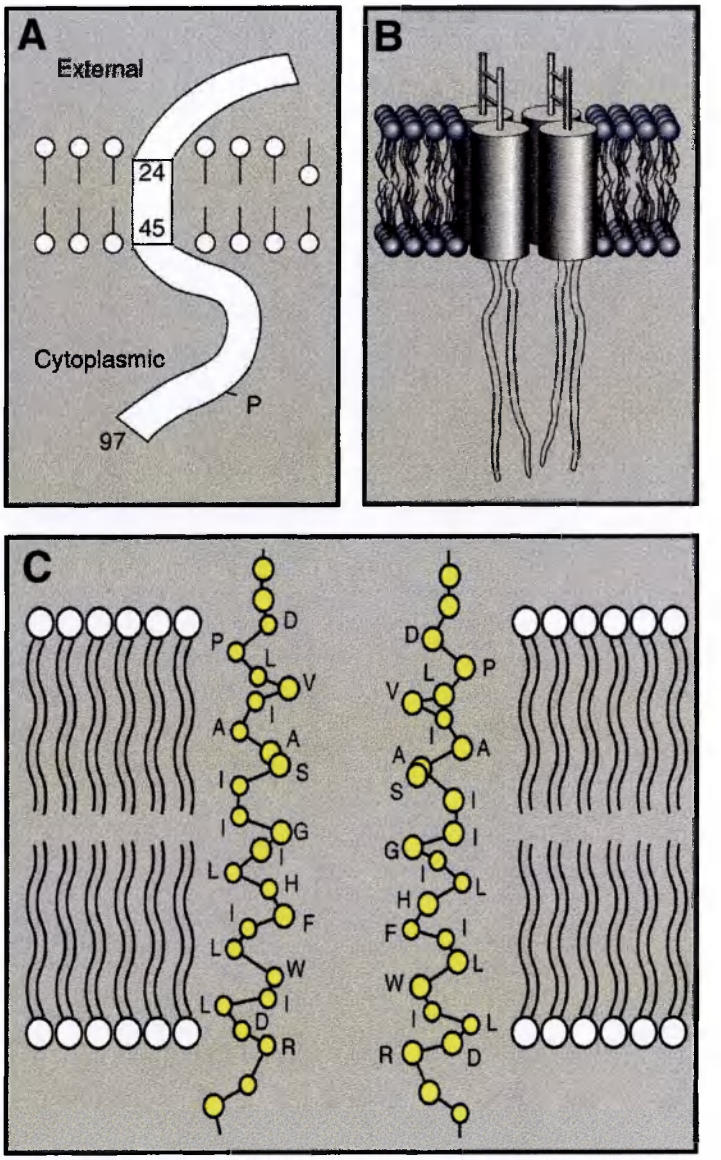
1.2.10 Matrix Protein (M1, M2)

The matrix gene of influenza A is coded by gene segment seven and represents a highly conserved bicistronic gene (203). It codes for two proteins; one open reading frame codes for the matrix (M1) protein and the other for the M2 protein in a +1 reading frame (204). The M1 protein represents the most abundant protein found in the influenza A virion, forming a matrix the lies between the lipid membrane and the ribonucleoprotein (RNP) core (402). M1 protein plays a role in regulating the switch from RNA replication to virus maturation (402, 404). Studies have demonstrated this by providing evidence that M1 protein inhibits the transcription activity of the RNP-associated RNA polymerase (133).

The potential for a second coded matrix polypeptide was discovered when a second open reading frame was found within the matrix gene (4, 200). The influenza M2 protein is abundantly expressed at the infected cell surface (161), although M2 is greatly underrepresented in virions with as few as 23-60 molecules incorporated into virus particles (411). M2 is a 97 amino acid integral membrane protein consisting of a disulphide-linked homotetramer (348). It comprises three domains, a 24 amino acid N-terminal extracellular domain, a transmembrane domain of 19 amino acids and a cytoplasmic tail consisting of 54 amino acids (Figure 1.3) (136).

The M2 protein of influenza forms an ion channel within the lipid envelope of the virus (Figure 1.3) (282). The discovery of the ion channel activity of M2 was a consequence of investigations with the anti-influenza drug, amantadine (346). M2 predominantly forms proton channels promoting the transfer of H^+ ions (281). Electrophysical measurement studies carried out using M2 expressed in *Xenopus laevis* (282), expressing M2 in mammalian cells (46), and by incorporating M2 into planar lipid membranes (360) have demonstrated the ion channel activity of M2. It has been shown that M2 ion channel has high selectivity for H^+ ions and low permeability for other cations and anions e.g. K^+ and Cl^- (46).

FIGURE 1.3 The influenza A M2 protein



The structure of the protein is illustrated as (A) the 97 amino acid monomer, (B) the tetramer structure that forms a channel in the virus membrane and (C) the hydrophobic transmembrane domain of the protein spanning the virus membrane. Adapted from (202) and (348).

M2 has been shown to play a functional role in two stages of the replication cycle of influenza (136). Each stage has been shown to be related to the functionality of haemagglutinin (139). After attachment and internalisation of the influenza virion, proton pumps cause a gradual reduction in endosomal pH. When internal conditions reach between pH 5 and 6, they trigger a conformational change in the haemagglutinin which promotes fusion of viral and endosomal membranes (106). As a result of lowered endosomal pH, the M2 ion channel opens allowing H^+ ions to penetrate the virion, thus lowering the pH. This causes a dissociation of the M1 protein structure, liberating the RNP for transport to the nucleus.

In certain pathogenic avian strains of influenza, e.g. fowl plague virus, M2 has a second role in modifying the pH of the TGN (346). Within the lumen of cisternae and vesicles of the TGN, acidic conditions are maintained by cellular proton pumps which transport H^+ ions into the lumen. The fusion activity of these avian HAs is proteolytically activated by proteases such as furin (341). This occurs in the TGN and renders them vulnerable to structural changes on exposure to low pH (157). Electron microscopy studies with amantadine have shown that the direct consequence of avian HA being exposed to pH lower than pH 5 is not preventing the formation of budding particles, but blocking their release (346).

The function of the M2 ion channel is discussed in greater detail later in Chapter 4, section 4.1.4.

1.3 Infection and disease

Influenza is generally a self-limiting disease of the upper respiratory tract in humans. The most common route of transmission person to person is through respiratory secretions. Infectious virus particles are spread through droplets of several microns in diameter expelled from coughing and sneezing or by the production of finer particle aerosols (3). There is also evidence that influenza can be transmitted by indirect contact *via* contaminated surfaces (351).

Infection is initiated in the upper regions of the respiratory tract, primarily the tracheobronchial epithelium, and then gradually spreads to the rest of the respiratory tract. The replication cycle of the virus lasts approximately four hours,

whereby progeny virions are released from infected cells and capable of infecting surrounding cells, and so the cycle continues. The incubation period of the virus can range from 1-7 days but on average lasts approximately 2-3 days (260). Titres of virus found in respiratory secretions can be high and this, coupled with the short incubation period and relatively long periods of virus shedding, has resulted in a highly infectious virus. Influenza can rapidly spread through a population with devastating effects as demonstrated in frequent epidemics and less frequent but more severe pandemics that have occurred over history (62).

The onset of illness for influenza A gives rise to symptoms including malaise, feverishness, chills and headache. The onset of disease is usually accompanied by a non-productive cough and such symptoms may last for 3-4 days but cough and malaise might persist for 1-2 weeks (260).

Complications associated with typical influenza infections are rare but individuals within at risk groups e.g. the elderly and those suffering from respiratory or other chronic medical conditions, are at greatest risk for developing complications (259). These may include acute bronchitis (56), otitis media (108) and pneumonia (219). Influenza viruses have been associated with croup (laryngotracheobronchitis), especially influenza A which appears to cause more severe disease than influenza B or C (344). Neurological complications have been associated with influenza infections, the most common ailment in children is Reye's syndrome. This presents as an acute encephalopothy that has been linked with children taking aspirin during, or after, an influenza infection (60, 241). This complication has been associated mainly with young children infected with influenza B, which is the primary viral cause of the syndrome (58).

1.4 Immunity

1.4.1 Humoral Immunity

The immune response initiated by the body following the invasion of influenza virus particles takes on two forms, neutralising antibodies and a cell-mediated response. The neutralising antibody response represents the first line of specific defence against the virus. Evidence for this immune response was first discovered

by studying the disease in ferrets where infected ferret serum neutralised virus in test animals (335). The primary neutralising antibody response against influenza infection is directed against the main surface antigen HA (372) although anti-neuraminidase (188) and anti-M2 (362) antibodies have also been implicated in the antibody response against infection. The immunity provided by serum IgG antibody is short lived due to pressure exerted on the antigenic regions of the HA molecule, specifically the HA1 domain which drives it to mutate and therefore escape the neutralising effect of the antibody. This ensures that infection with one type or subtype of influenza does not provide protective immunity against others.

1.4.2 Cellular Immunity

A second form of immunity provided is cell mediated. Cytotoxic T cells can provide protection against influenza but unlike neutralising antibody they can provide cross-reactivity between different influenza A strains (419). The cross-reactive antigens recognised by the cytotoxic T cell repertoire appear to be the conserved internal proteins of influenza A viruses, e.g. the nucleoprotein (176). T-helper cells recognise peptide fragments of the virus in association with major histocompatibility complex class II molecules on the surface of antigen presenting cells.

Other parts of the immune system complete the overall response to an influenza infection; these include complement, natural killer cells and macrophages. Without neutralising antibody and cell mediated response these aspects of the immune system can not cope but together all form a good immune response.

1.5 Seasonality

Influenza virus circulates within a given population within defined seasons every year. This circulation occurs in the winter months in the Northern Hemisphere and in the wet season of Southern Hemisphere countries. The factors limiting influenza to these defined periods are still not well understood although social behaviour and climate are thought to play a part. Epidemics of influenza activity occur annually or every few years due to gradual genetic changes within the virus

although pandemic activity is much more severe in nature but less frequent and unpredictable.

1.6 Genetic Diversity

1.6.1 Antigenic Drift

Influenza is a negative-stranded RNA virus that requires the presence of an RNA-dependent RNA polymerase to produce mRNA from a negative-sense template. The RNA polymerase molecule is highly error prone because it lacks 3' to 5' exonuclease activity, the proof-reading function of viral replication (338). The high mutation rate of the RNA polymerase, from 1×10^{-3} to 1×10^{-5} misincorporations per base site per round of copying (78), causes a build up of mutations within an influenza population. If any mutation confers a selective advantage then there may be a rapid build up of a variant population. This accumulation of mutations results in a gradual genetic change, or drift. If changes have occurred in the antigenic surface glycoproteins (HA or NA) then the virus may acquire a subtly different immunogenic profile, sufficient to evade the humoral antibody response and to reinfect. This phenomenon is described as "antigenic drift" and is responsible mainly for interpandemic periods of influenza activity causing large epidemics and localised outbreaks.

1.6.2 Antigenic Shift

The multipartite genome allows reassortment of two influenza viruses to occur. This genetic reassortment of dual-infecting viruses within a host cell has the potential to produce progeny virions with novel constellations of surface and internal proteins (317). This process is termed "antigenic shift". This causes the sudden appearance of new viruses into the human population for whom the majority have no immunological memory to the novel antigenic subtype. This produces the infrequent severe influenza activity termed a "pandemic".

1.6.3 RNA Virus Populations as Quasispecies

It has been proposed that RNA viruses exist in complex populations that can evolve rapidly (160). Eigen *et al.* (1981) introduced the term "quasispecies" to refer to diverse RNA populations that had been studied and were observed to be undergoing constant change (83). Since the introduction of the term quasispecies, there has been some confusion to its exact definition and the word has been used in a variety of different ways. The widely accepted definition is that a quasispecies is a population of viruses containing diverse variants which are all phenotypically related but carry distinct genomic sequences as a result of mutation, drift and the impact of selection (333). However, several studies have confused the issue by using quasispecies to refer to each of the distinct virus sequences that together make up the quasispecies (92). In this study, quasispecies will be used to describe a population of phylogenetically related variants within an individual, i.e. a single strain of virus containing a heterogeneous population of variants.

The existence of quasispecies can be attributed to the genetic make-up of the viruses concerned. Mutant genomes are continuously generated in RNA viruses as a direct consequence of high mutation rates during viral replication (77). The RNA polymerase molecule found in influenza and other RNA viruses lacks 3' to 5' exonuclease proofreading activity, the result of which is a high rate of mutation of different genes (338). Mutation rates of RNA viruses during replication can be in the range of 1×10^{-3} to 1×10^{-5} misincorporations per base site per round of copying (78) compared to the low mutation rate in DNA replication that can be as low as 1×10^{-8} to 1×10^{-11} (159).

Within a quasispecies there appears to be a process of continual selection which has been compared to the concepts of Darwinian evolution (75). Selection pressures create a quasispecies that is stable until mutation happens to produce a new sequence that allows the variant to dominate over other existing sequences within the quasispecies e.g. through increased growth rate. When this happens, the new "fittest" sequence succeeds until it dominates and the old quasispecies disappears (83). The potential to generate new variants is a key feature of viral quasispecies and allows the virus to evolve at a fast rate. This is especially significant for influenza viruses where even in the absence of immune pressure, normally the driving force behind antigenic drift, the virus can undergo antigenic

variation due to mutational pressure (75). This antigenic variation in the absence of immune pressure has been termed the "random change" model. It is proposed that antigenic sites located on the surface of envelope proteins will be subjected to less stringent structural requirements than domains involved in interactions with other structural proteins (76). Therefore, substitutions at these sites will tend to be represented more in the spectrum of mutants within the quasispecies (75).

The fitness of a virus or a particular genotype within a quasispecies is the measure of the individual's ability to survive and reproduce. The success of an individual in evolving to gain fitness is determined not by absolute fitness, but relative fitness compared to other genotypes within the quasispecies (217). In order to increase fitness, mutations arising in the genotype must confer some sort of advantage to the carrier. The majority of new mutations arising in a quasispecies reduce the fitness of the carrier and are eventually removed from the population, this is classed as negative selection. A mutation arising may confer a genotype with fitness equal to the dominant mutation in the population, and this mutation is classed neutral. Occasionally, a mutation may arise that confers a selective advantage to its carrier enabling the individual to gain fitness with respect to the other genotypes present in the population; this is positive selection.

Therefore, it can be concluded that by way of a polymerase molecule that is error prone, RNA viruses are subject to high mutation rates and as a result exist as dynamic changing populations termed quasispecies. Through random change or when a selective pressure is exerted on the quasispecies, the distribution of genotypes within the population can be altered with dominant genotypes succeeding and displacing other variants to become the fittest genotype. An important source of data concerning the evolution of quasispecies can be obtained from individuals suffering with persistent infections. Several studies have discovered that in immunocomprimised patients, where persistence is as a result of lowered immunity, the viruses isolated can be genetically different due to random changes rather than immune pressure (90, 193, 304). These studies provided good models for investigating the dynamics of quasispecies. The fitness of different genotypes within quasispecies can be quantified using growth and competition assays, both of which will be discussed in greater detail later.

1.7 Surveillance of influenza

Influenza activity in the UK varies considerably from year to year, a trend that is observed world-wide. During major epidemics a substantial proportion of the population can be affected and illness may be clinically severe in many. This may have important social and economic impacts on the infrastructure of the country and therefore surveillance of influenza activity during seasonal periods is essential. The World Health Organisation (WHO) global influenza surveillance monitors influenza activity on a world-wide basis. Data are collated from a network comprising four international centres in London, Atlanta, Melbourne and Tokyo and 110 national laboratories in 83 countries (391). Epidemiological data, antigenic and genetic composition of recently isolated strains and data on the ability of the current vaccine to evoke antibody responses are all assessed to aid the prevention and control of influenza.

Within the UK, several indicators are used to quantify the level of influenza activity within the population which, when considered together, describe the features of influenza activity each year. Data for influenza and influenza-like-illness (ILI) are collected in the sentinel general practice network of the Royal College of General Practitioners (RCGP) in England and Wales. Each week, new episodes of illness are calculated and the incidence rates calculated per 100,000 population (87); this weekly measure of activity is referred to as the RCGP index. The index provides a tool to compare the influenza activity of different years and to identify epidemic activity (66-68, 70, 169, 170, 389). To aid the interpretation of influenza activity, defined thresholds have been implemented that identify baseline (<50 cases of ILI per 100,000 population), normal (50-200 per 100,000 population), high (200-400 per 100,000 population) and epidemic (>400 per 100,000 population) influenza activity (69). Although limited in the amount of virological data that can be extrapolated from them, they represent a simple and logical representation of influenza activity.

1.8 Prevention and treatment of influenza

1.8.1 Vaccination

The earliest vaccinations were performed by simple injection of live virus into human subjects who developed immunity to subsequent challenges (102). The first inactivated vaccine was produced in 1937 (336) but it was not until 1943 that the first vaccine trials were performed in the US showing that the newly developed influenza vaccine was approximately 70% effective at protecting subjects from infection. Larger trials were performed on US army recruits which provided more evidence that the vaccination afforded personnel with consistent protection (101). This eventually led to the licensing of the first influenza vaccine in the USA in 1947 (399).

Since these early vaccine trials there have been several developments that have improved the overall efficacy and tolerance of influenza vaccines. Currently, influenza vaccines are manufactured as trivalent vaccines containing two type A viruses and one influenza B strain (60). The vaccines are inactivated and highly purified, cultured in pathogen-free embryonated hens' eggs. There are four types of vaccine of which two are currently available, the split or disrupted particle and sub-unit or surface antigen vaccines (72). Vaccines are administered as an injection by the intramuscular route with generally one dose for adults and two for children less than twelve years of age. The overall efficacy of vaccines in healthy adults is approximately 70-90% but is often lower in certain groups such as the elderly and young children (60).

Current research, which is shaping the future of vaccine production, has recently had many encouraging breakthroughs. Cold-adapted vaccines have been used successfully in the former USSR and Russia for the past 30 years (185). They utilise a donor strain passaged in tissue culture at 25°C which becomes attenuated in humans. The vaccine strain is created by producing a reassortant cold-adapted virus presenting the surface antigens of the desired vaccine strain. The resulting virus induces longer lasting immunity than killed virus vaccines. Clinical trials in the USA have established the safety, immunogenicity and efficacy of cold-adapted live attenuated vaccines using A/Ann Abor/6/60 and B/Ann Arbor/1/66 as donor strains in children, young adults and the elderly (24, 254, 258).

To improve the delivery of influenza vaccines, and therefore increase the immunological response, research has been carried out into liposomes which act as adjuvants for the vaccine antigen. Haemagglutinin and neuraminidase preparations have been incorporated into liposomal structures (virosomes) and tested in preclinical investigations. In the animal model these virosomes stimulated increased primary and secondary antibody responses when compared to original antigen preparations (120). Clinical trials of these vaccines in human subjects have demonstrated that immunogenicity in the host was significantly better than existing vaccines (55, 109).

The other main area of research into novel vaccines involves DNA vaccination. The principle is to insert the DNA coding sequence of antigen of choice into a bacterial plasmid that is then delivered into the body, usually muscle, where the antigen is expressed from the plasmid and induces an immune response (381). Early investigations using animal models showed potential with DNA plasmids expressing HA eliciting protection to mice against lethal challenges with influenza virus (303). A similar investigation using a plasmid encoding influenza A nucleoprotein also provided mice with protection against influenza infection (363). Human trials with DNA vaccines have not yet started because there are still certain safety concerns. Anti-DNA antibodies, host immune status and transformation of cells into tumours must all be considered and investigated before any DNA vaccine can be injected into human subjects (380). The delivery of DNA vaccines with the help of adjuvants has been investigated. It has been found that adjuvants enhance the efficiency of influenza DNA vaccines; trials in mice using a cytokine gene as an adjuvant significantly improved the protection conferred by the vaccine (213).

1.8.2 Antiviral drugs

The potential threat of future influenza pandemics and the continuing generation of drug-resistant viruses to current compounds leave no doubt that the development of new antiviral drugs against influenza is required. Antiviral drugs have been, or are in the process of being designed to act against virtually every stage of the influenza infectious cycle. An obvious target is during attachment of influenza virus particles to the target cell. There are two possible mechanisms to achieve this, 1)

by forming complexes with sialic acid receptors on target cells rendering them unavailable to haemagglutinin, or 2) by blocking the binding domains of the haemagglutinin molecule with a compound that complexes with sialic acid on the cell surface binding site. Research into sialic acid derivatives has produced compounds with varying potencies. The most potent compounds to date have been polyacrylamide derivatives incorporating sialic acid that bind to and block the receptor-binding sites on the haemagglutinin (214, 231). Published data concerning these, and other inhibitors of viral attachment, have involved *in vitro* testing and as yet no data on *in vivo* studies have been published (214, 231).

The stage of virus entry presents a major problem in the development of inhibitors. Influenza viruses enter the target cell after attachment by a process of host cell-mediated endocytosis. A compound targeting this would be very difficult to develop because this represents an important function of the host cell (207). During the process of virus uncoating, there are two stages that can be targeted. Firstly, during membrane fusion between viral and endosomal membranes, and secondly, the release of RNP by M1 allowing its migration to the nucleus (379).

A series of compounds, sulfonic acid polymers, were shown to be inhibiting towards influenza A in tissue culture. These compounds proved to be potent and selective when administered to immunocompetent mice and the mode of antiviral activity was determined to be inhibition of virus-cell fusion (171).

In order for virus replication to occur, release of the ribonucleoprotein (RNP) from M1 is essential. The antiviral drugs amantadine and rimantadine both affect the M2 ion channel, blocking the flow of H^+ ions and therefore inhibiting the release of the RNP (137). The majority of influenza antiviral research over the past two decades has focused on amantadine and rimantadine; these two compounds are discussed in section 1.8.3.

The preparation of anti-sense oligonucleotides has provided the means to block the copying of influenza virus. These short sequences of nucleotides can bind to the RNA polymerase and specifically inhibit cap-dependent mRNA synthesis (207).

Ribavirin, a synthetic nucleoside analogue has been shown to have antiviral activity for several viruses including HIV, herpes simplex viruses and influenza A and B. It is thought to act by suppressing the synthesis of viral nucleic acid by reducing available guanosine triphosphate. Ribavirin has also been shown to have a direct effect on the viral polymerase (267). A number of clinical trials have demonstrated the effectiveness of ribavirin at reducing the severity of influenza virus infections in patients given the drug prophylactically, however, it had no effect on the manifestation of mild illness (229). The use of ribavirin is limited due to its administration, requiring lengthy therapy and hospitalisation.

For the remaining stages of the infectious cycle, i.e. secondary transcription, replication of vRNA, translation of mRNA, post-translational modification of viral proteins, and assembly and release of progeny virions, there are surprisingly few viral inhibitors that have been developed (207).

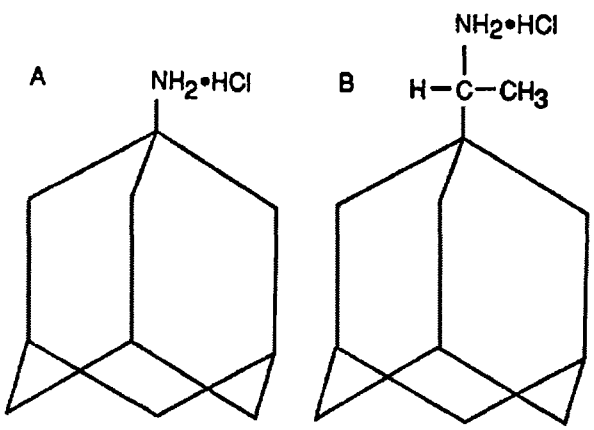
1.8.3 Amantadine and rimantadine

The antiviral activity of amantadine was first reported by Davies *et al.* (1964) in a series of experiments that demonstrated the inhibition of influenza infections in tissue culture, chick embryos and mice by amantadine (65). This work was followed by numerous other studies investigating the properties of amantadine. In 1966 the drug was licensed for clinical use but only against influenza A subtype H2N2 (249). The following years saw the disappearance of H2N2 and the emergence of H3N2 subtypes which caused severe pandemics (52). Amantadine could not be legally used against these new influenza A viruses until 1976 when the drug was relicensed for all influenza A type viruses (249). Early studies concluded that amantadine prevented the adsorption of influenza to target cells (65) but it was not until approximately two decades later that the target protein of amantadine in influenza A was discovered (142).

1.8.3.1 Structure

Amantadine is a symmetrical, thermally stable amine with a cage-like structure (Figure 1.4) (8). After the initial synthesis of amantadine in 1963 studies were carried out to discover more potent agents with a broader spectrum of action. Amino groups and other sites were substituted on the amantadine molecule and structural variations of the drug were developed and tested. It resulted that very few of these congeners had any useful antiviral activity against influenza. However, a small group of derivatives, where substitutions on a specific site had been performed, demonstrated increased potency against influenza, and one of these drugs was rimantadine (2). The structures of these two compounds are illustrated in Figure 1.4.

FIGURE 1.4 Influenza A anti-M2 protein drugs



The structures of (A) 1-amino adamantane hydrochloride (amantadine) and (B) α-methyl-1-adamantane methylamine hydrochloride (rimantadine) are Illustrated. Taken from (136).

Amantadine and rimantadine inhibit two stages of the infectious cycle of influenza A virus (136). Early studies revealed that the drugs had no direct inactivating effect on virus infectivity or adsorption to target cells but uncoating of virus was markedly inhibited by amantadine (179). It is now known that amantadine prevents initiation of primary transcription as a direct result of inhibiting the dissociation of M1 and

RNP (137). The drugs act as allosteric blockers (8), which appear to bind to the M2 protein causing a conformational change within the pore-forming region. This inhibits the flow of protons through the ion channel and stops acidification of the virus interior (283). The consequence of this is that the RNP is not transported to the nucleus and hence transcription, translation, and assembly of progeny virions cannot occur.

Amantadine is also known to affect certain pathogenic avian strains of influenza A virus as an indirect consequence of inhibition of M2 function (136). Where intracellular proteolytic cleavage of HA0 to HA1/HA2 occurs, it renders the virus sensitive to the late action of the drugs (136). Amantadine blocks the M2 channels which causes an increase in acidity within the TGN and triggers the irreversible conformational change in HA. As a result, release of virus is prevented and virus replication inhibited due to the expression of the low pH form of HA on the surface of the virus (310). The antiviral activity of amantadine and rimantadine is discussed in greater depth in Chapter 4, section 4.1.5.

1.8.3.2 Clinical Efficacy of Amantadine and Rimantadine

The effectiveness of amantadine and rimantadine for both the prophylaxis and treatment of influenza infections has been established through many studies over the past 30 years (37, 74, 149, 165). The benefits of prophylactic treatment have been proven using experimental challenges of healthy individuals with influenza A (300). Vital dose-response studies have helped determine the optimum dose for protection; the drugs have a low toxic-to-therapeutic ratio that makes dosing vitally important (8). In naturally occurring influenza infections in adults and children, amantadine and rimantadine have been shown to have an efficacy of up to 90% in preventing influenza illness (37, 61). Within closed populations, e.g. boarding schools, nursing homes, prisons etc, the drugs have also been shown to be effective in preventing illness, and in some cases reducing mortality (14, 275).

Amantadine and rimantadine have proven equally effective in treating acute influenza infections in healthy adults, children and the elderly (26, 130, 152). The drugs have an equal efficacy compared to the annual vaccine and are able to

prevent illness or reduce influenza-like symptoms. However, it is thought that administration of amantadine and rimantadine must be initiated within 48 hours of the onset of symptoms if they are to be effective.

1.8.3.3 Disadvantages of Amantadine and Rimantadine

Until 2000, amantadine and rimantadine were the only antiviral drugs licensed for the treatment of influenza. They have been proven to be potent inhibitors of influenza and reduce disease but their use in the UK has been limited. The main limitation in their use is that they target the M2 ion channel and therefore are inhibitory only to influenza A viruses which possess the protein. Infections caused by influenza B or C virus cannot be treated with these drugs. Gene segment six of influenza B and C codes for the NB and CM2 proteins respectively (164, 323). These proteins have been shown to be analogous to the influenza A M2 protein in respect to size and post-translational modifications (218, 394), and the NB protein has been shown to form ion channels (349). However, the replication of influenza B and C viruses is not selectively inhibited *in vitro* or *in vivo* by amantadine or rimantadine (138).

Another major drawback that has limited the use of the drugs is the emergence of resistance. Influenza viruses resistant to amantadine and rimantadine can be readily isolated *in vitro* by growing the virus in the presence of drug (10). Data from animal models have suggested that virulence, pathogenesis and transmissibility of amantadine- and rimantadine-resistant viruses are unaltered compared with wild-type sensitive virus (22, 350). Studies with avian models demonstrate that resistant viruses can be readily detected in birds and that the virus is both virulent and transmissible to other birds (22). In the ferret model, inoculation of resistant viruses demonstrated their unaltered virulence and growth characteristics (350). It has been shown that viruses display cross-resistance to both amantadine and rimantadine (23).

It is well established that the principle determinant of amantadine-resistance and the target of the drug is the M2 protein (137). Comparisons of sequences of the M genes of resistant and sensitive viruses show that nucleotide changes within the M

gene result in an amino acid change in the M2 protein (25). The position of these mutations are downstream of the termination site for translation of M1 and therefore it is only the sequence encoding M2 translated from the spliced mRNA that is altered (137). The genetic basis for resistance is single amino acid changes at one of five positions within M2 (118, 142). Resistance to amantadine and rimantadine will be discussed in greater detail in Chapters 3 and 4.

The emergence of resistant epidemic strains is, in theory, a possibility. If an M gene coding for resistance was introduced into a location where an epidemic strain was emerging, then it is possible that the resistant virus could disseminate widely (144). However, it is unknown whether the resistant strain would be stable enough to survive without selective drug pressure and whether it would be able to compete with the circulating wild-type virus. The relatively limited use of amantadine (25) may not be sufficient to exert enough selective pressure on the virus to cause substantial transmission of resistant virus during an epidemic.

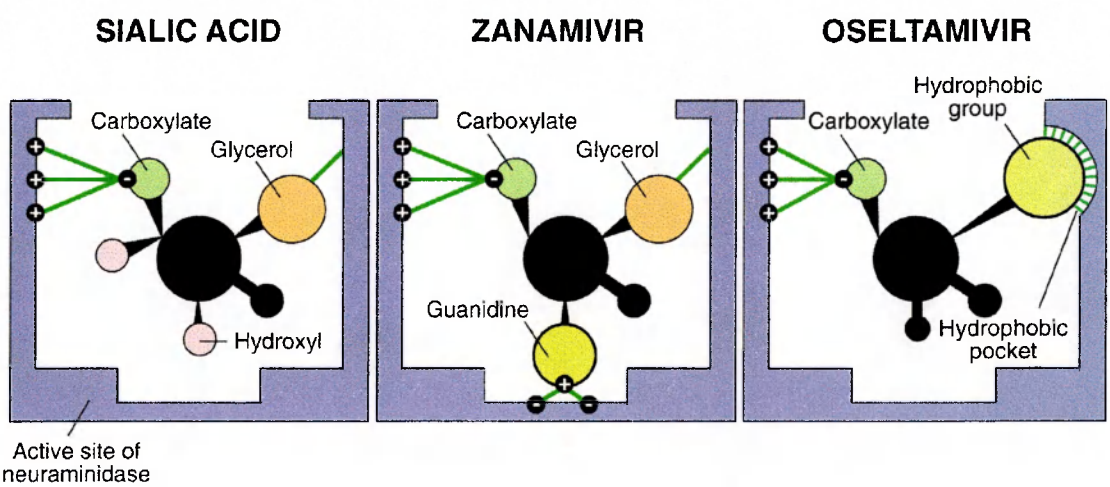
When amantadine and rimantadine were first licensed, there were initial fears concerning side effects experienced by patients undergoing treatment. It has been reported that these occur in approximately 6% of patients, more often in the elderly and include neurological reactions, light-headedness and an inability to concentrate (25, 136). Due to the lower incidence of neurological side effects associated with rimantadine treatment, it is now perceived to be the drug of choice in some countries, although it can still cause gastro-intestinal complaints and nausea and vomiting in some cases (8). In general, there seems to be a lack of awareness of the potential of these drugs for preventing or limiting influenza A infections.

1.8.4 Neuraminidase Inhibitor Drugs

Early attempts to utilise the neuraminidase surface glycoprotein as a target for antiviral therapy were made by trying to synthesise analogues of sialic acid (Neu5Ac) as an antiviral agent (82). These experiments had little success in demonstrating any antiviral activity but gradually more potent molecules were developed. It was not until the mid-1970s that the actual mode of action of sialic acid as a neuraminidase inhibitor (NI) was discovered. It was demonstrated by

electron microscopy that by adding analogues of sialic acid to virus cultures, newly synthesised progeny virions were not released from the surface of the host cell (271). Particles would accumulate at the cell surface and released virions formed large aggregates mainly through virus-virus and virus-cell interactions (271). The next major breakthrough in the development of the drugs came with nucleotide sequencing and X-ray crystallography studies of the neuraminidase molecule (54, 369). This allowed a greater understanding of the structure of the enzyme and its active binding site and further encouraged the development of new sialic acid derivatives with greater antiviral properties. One such group of compounds was reported by von Itzstein *et al.* (1993) and included 4-guanidino-Neu5Ac2en, zanamivir (374). Experimental evidence showed that the OH group found at carbon atom 4 in the structure of this compound was positioned close to the active site on the NA molecule (Figure 1.5). Substituting the OH group with a guanidino group resulted in an increase in antiviral potency (374). Zanamivir has been developed by Glaxo Wellcome under licence from the Australian company, Biota Holdings.

FIGURE 1.5 Diagrammatic representation of neuraminidase inhibitor compounds blocking the active site of the NA molecule



Green lines represent bonds formed between different groups on the drugs and amino acids within the site. Zanamivir and oseltamivir bind to the site more readily than sialic acid through a guanidine and hydrophobic group respectively, increasing their antiviral potency. Adapted from (210).

During all studies and trials involving zanamivir attempts have been made to monitor levels of resistance. *In vitro*, the levels of resistance are low compared to similar studies with anti-M2 protein drugs. Resistant viruses emerge after one or two passages in the presence of amantadine and rimantadine (268). Resistant viruses have been isolated by passaging in the presence of zanamivir but resistance is significantly slower to develop compared with the anti-M2 protein drugs (278). Mutations that reduce the sensitivity of influenza A and B viruses to neuraminidase inhibitors have been mapped to the active site of the neuraminidase (128, 243). These mutations interfere with the action of sialic acid analogues inhibiting the release of progeny virions from the infected cell. Mutations conferring resistance have also been mapped to the haemagglutinin molecule (124, 242). Mutations within the haemagglutinin are thought to affect the affinity or specificity of haemagglutinin binding reducing the dependence on the neuraminidase for progeny release (242). To date the generation of resistance in humans has not been readily documented. One of the few cases observed involved the isolation of a resistant influenza B virus from an immunosuppressed patient (127). The virus isolated had mutations within both the haemagglutinin and neuraminidase that reduced the sensitivity of the virus to zanamivir by 1000-fold. Compared to amantadine and rimantadine where resistance has affected administration of the drugs, the resistance frequency of zanamivir is very low and it is thought that this will not affect the clinical usefulness of the drug (125).

Phase I and II clinical trials have proven the potency and safety of zanamivir and phase III trials have proven its efficacy in human subjects. The trials have consisted of randomised, double-blind placebo controlled studies of large groups of subjects in different locations. Results from trials in both the Northern Hemisphere (150, 153, 230, 250, 252) and the Southern Hemisphere (228) provided the evidence required to demonstrate the clinical usefulness of the drug. The main conclusions from these studies were that in subjects treated with zanamivir, symptoms of headache, sore throat, fever, cough, weakness and muscle ache were alleviated faster than placebo groups. This was 1 to 2.5 days between treated and placebo groups but for the drug to be clinically effective it had to be administered less than 30-40 hours after the onset of symptoms. The incidence of complications and use of antibiotics was also reported to be lower in treated groups. Side effects were described in approximately 3% of subjects, the most common being sinusitis, diarrhoea and nausea, but this was comparable to

placebo groups. Zanamivir is applied topically to the respiratory tract, patients orally inhale the drug using a Diskhaler (252) but it has been reported that this can cause bronchospasm and/or a decline in lung function in some patients with underlying respiratory disease (395).

Another promising NI drug, oseltamivir (Tamiflu) was first described in 1997 (190) and was licensed in the US in late 1999. The main advantage oseltamivir has over zanamivir is that the bioavailability of the drug is much greater and it can be taken orally. The efficacy and safety of oseltamivir has been proven and the results suggest that the drug is effective at reducing the duration and severity of illness (146, 154, 245, 261, 361). Oseltamivir significantly reduced the duration of illness in infected patients by 25% and if treatment was initiated within 24 hours of the onset of symptoms the reduction in duration of illness was further reduced to 37%. It was reported that the overall health of treated patients was improved in respect of symptoms, activity and sleep quality with secondary complications including sinusitis, bronchitis and pneumonia also reduced. The overall conclusions from the above studies are that oseltamivir is well tolerated and treatment reduces the severity and duration of acute influenza infection.

Current research is continually developing new NI drugs. One such compound, RWJ-270201 is produced by BioCryst Pharmaceuticals and is currently undergoing *in vitro* and *in vivo* trials (16, 330, 332). Preliminary results from these early studies suggest that human volunteers infected with influenza show significant reductions in virus titres after treatment with the drug. Further trials will be required to determine the full clinical effectiveness of the drug before it appears on the market.

1.9 Objectives

The main objective of this thesis is to investigate the relationship of the evolution of influenza A matrix genes to their functional properties. The main function of one matrix gene spliced product, the M2 protein, is to regulate ion exchange across the influenza A virus envelope. This function has been shown to inhibited by the anti-influenza drug amantadine.

The first set of objectives is to study the M2 protein of a large collection of influenza A viruses that circulated the UK general population from 1958-1999. The scale of the study has not previously been performed in respect to the number of viruses and years of circulation. It is made especially unique because the viruses in this study have circulated a geographically isolated population. It is important to assess the impact of antiviral drugs on a population and to identify causes of drug-resistance. There are several aims to this part of the work:

- Instigate methods and protocols to assess the susceptibility of influenza A viruses to amantadine by phenotypic and genotypic methods
- Estimate the frequency of amantadine-resistance in influenza viruses of H3N2, H2N2 and H1N1 subtypes that circulated the UK from 1958-1999
- Sequence the M2 protein of resistant viruses to determine the underlying molecular changes responsible for reducing the sensitivity of virus to drug
- Assess the frequency of amantadine-resistance mutations occurring within the population
- Sequence a representative number of amantadine-sensitive viruses over an equivalent time period to analyse the drug genotype of these viruses
- Estimate the rate of evolution of the matrix gene and its' spliced products (M1 and M2) and relate findings to functional properties of the genes

The second set of objectives is to involve the study of a set of viruses obtained from a persistent infection of an immunocompromised patient. Twelve viruses were isolated over a period of eighteen months and represent a unique opportunity to study the progress of viral population dynamics and evolution over the period of infection. The main objectives in this research are:

- Characterise each isolate phenotypically in respect to matrix gene function
- Sequence the matrix gene products of each virus to evaluate changes in genotype over the persistent infection
- Assess changes in the antigenic profile of each virus to evaluate selective pressures presented in the host
- Estimate the rate of evolution of the matrix and haemagglutinin genes from the persistent infection and compare to representative field strains

Therefore, it is proposed that the work is unique in two ways. Firstly, it represents the largest study of amantadine susceptibility in influenza A viruses to date, and is the first major assessment of resistance in the UK. With the predicted increase in use of influenza antiviral drugs, this kind of data is essential in understanding the mechanisms of emerging resistance. Secondly, the study of a persistent infection, to our knowledge the longest period of influenza persistence recorded to date, will enable a unique opportunity to study viral evolution within a single host. The two sets of data will complete a detailed analysis of matrix genes and relationships to functional properties in both a large population and a single host.

Chapter 2

Materials and Methods

2.1 Virus Stocks

The majority of viruses used in the study were originally isolated from clinical respiratory specimens in mammalian tissue culture (>75%) while viruses isolated prior to 1985 were isolated in the allantoic cavity of fertile hens' eggs. Viruses were stored at –70°C or freeze-dried at time of isolation. Freeze-dried material was reconstituted in 1 ml phosphate buffered saline (PBS) and inoculated into Madin-Darby canine kidney cells (MDCK), which were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK).

2.2 Cells

Confluent MDCK monolayers were washed twice with PBS and then rinsed with trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA; Life Technologies Ltd, Paisley, UK). Excess trypsin was removed and the monolayer incubated at 37°C for 15 min. Cells were resuspended in minimal essential medium (MEM) with Earle's salts and 25 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid] (HEPES; Life Technologies Ltd) with 10% fetal bovine serum (FBS; Life Technologies Ltd) supplemented with gentamycin sulphate (50mg/ml; Biowhittaker, Walkersville, Maryland, US). Cells were incubated at 37°C until confluent and maintained at 33°C in MEM until required. MDCK cells were subcultured with a split ratio of 1:4. Primary rhesus monkey kidney cells (RMK; ECACC) were passaged using the above method and subcultured at a splitting ratio of 1:2 to 1:4.

2.3 Culture of Influenza Virus in Mammalian Tissue Culture

MDCK and RMK monolayers were subcultured into glass tissue culture tubes and incubated at 37°C until confluent. Cells were washed twice with PBS and 100 µl virus inoculum added to the tube. Two tubes, one MDCK and RMK were inoculated for each virus. An adsorption period of 1 hour was allowed during which the tubes were occasionally rocked to ensure the cell monolayers were covered by the virus inoculum. After the adsorption period, virus inoculum was removed from the tubes and 1 ml post-inoculation medium was added. Post-inoculation medium

was pre-prepared containing MEM supplemented with penicillin/streptomycin solution (final concentration 1%; PAA Laboratories, Austria) and 1 Tosylamide-2-phenylethyl chloromethyl ketone-treated (TPCK) trypsin (final concentration 1.25 mg/ml; Worthington Bioproducts Ltd., Lakewood, NJ, US). Tubes were incubated at 33°C on a roller for seven days during which each inoculated tissue culture was examined microscopically for the appearance of cytopathic effect (CPE). Haemagglutination assays (section 2.8) were performed on all cultures on day 7 to detect viral growth. Positive cultures were aliquoted and stored at -70°C, negative cultures were passaged into fresh tissue cultures and incubated for a further seven days. If no viral titre was detected after this further seven day incubation, the relevant tubes were discarded.

2.4 Culture of Influenza Virus in Fertile Hens' Eggs

Isolates that failed to demonstrate any growth in tissue culture, or viruses to be prepared for antigen controls were cultured in fertile hens' eggs (122). Sterile eggs were incubated in a humid atmosphere at 35°C for ten days. Prior to inoculation, each egg was individually candled to ensure that the chick embryo was alive and to locate the exact position of the air sac within the egg. The shell above the air sac of a fertile egg was removed using sterile forceps and to aid the visualisation of the internal components of the egg the membrane was swabbed with liquid paraffin. Stock viruses were diluted in saline broth (100 ml saline, 10 ml brain heart infusion broth, 100 µl gentamycin sulphate) and 200 µl inoculated into the allantoic cavity of the egg using a 0.6 x 25 mm gauge needle (Terumo, Belgium) taking care to avoid damaging any blood vessels or the embryo. The egg was sealed with tape and incubated in a humidified atmosphere at 37°C for 72 hours. Following incubation eggs were placed at -20°C for 1 hour to kill the embryo and the allantoic fluid harvested using a plastic pastette. A haemagglutination test was performed on all harvested fluids and the titre recorded. Viruses were either used immediately or aliquoted and stored at -70°C for future use.

2.5 Virus Titration

Viruses grown in tissue culture or fertile eggs were titrated using a plaque assay. MDCK cells were plated out into 12-well flat bottom microplates (Life Technologies) at a seeding density of 5 x 10^4 cells per well in MEM with Earle's salts and 25 mM HEPES (Life Technologies) containing 10% FBS (Life Technologies) and incubated for 3 days at 37°C with 5% CO_2 until monolayers were confluent. Virus dilutions were prepared by adding 100 µl of virus to 900 µl of virus transport medium (VTM) and then transferring 100 µl of this dilution to 900 µl of VTM. The serial dilution was continued until 1 x 10^{-6} and the last 100 µl discarded. Medium was aspirated out of each well and cells washed with PBS twice to ensure all serum from the medium had been washed away. To each well, 100 µl of corresponding virus dilution was added, each dilution had duplicate wells and two negative control wells containing 100 µl VTM were also included. Each plate was agitated every 10 minutes to ensure that the monolayer was covered by the virus inoculum at all times and virus adsorption was for a total of one hour at room temperature. To prepare the agar overlay, 2 ml aliquots of 5% indubiose (Biosepra, France) were heated in a boiling bath for approximately 15 minutes until the agar was molten. Overlay medium consisting of 100 ml MEM, 20 µl of DEAE dextran (10%; Pharmacia Biotech, Uppsala, Sweden), 400 µl of L-Glutamine (200 mM; Life Technologies Ltd), 1.0 ml of non-essential amino acids (Biowhittaker), 1.0 ml gentamicin sulphate at a working strength of 50 mg/ml, 5 ml of 7.5% sodium bicarbonate (Life Technologies Ltd), and 300 µl of TPCK-trypsin was added to 5% indubiose (Biosepra, Cergy-Saint-Christophe, France) in a 9:1 ratio respectively, allowed to cool sufficiently and then 1.5 mls added to each well. Once set, plates were incubated at 37°C with 5% CO_2 for 72 hours. After this time, cell monolayers were fixed with glutaraldehyde (SERVA, Heildelberg, Germany) and stained with carbol fuchsin solution (Merck Ltd, Lutterworth, UK). Virus infectivity titres were calculated from wells containing a dilution of virus that produced approximately 30 to 100 plaques using Equation 2.1:

EQUATION 2.1 Method for calculating virus infectivity titre (PFU/ml)

$$\text{Infectivity titre} = \text{plaque count} \times \frac{1}{\text{Dilution}} \times \frac{1}{\text{Inoculum volume}} \quad \text{PFU/ml}$$

2.6 Antiviral Susceptibility Enzyme-Linked Immunoassay (EIA)

MDCK cells in MEM (supplemented with 10% FBS) were seeded (5×10^3 cells per well) into 96-well sterile microtitre plates (Greiner Labortecknik Ltd., Stonehouse, UK) and incubated at 37°C with 5% CO_2 overnight or until cell monolayers were confluent. Viruses to be screened were serial diluted 10^{-1} to 10^{-3} in MEM, and in MEM with amantadine (final concentration 1.0 µg/ml; Sigma chemical Co., MO, US). Amantadine was used at this concentration to enable direct comparisons of the results obtained from this study with other published reports that had used similar methods with amantadine at 1.0 µg/ml (23, 25, 147, 417). Growth medium was removed from the wells, cells were washed once with PBS and blotted dry. Dilutions of virus were added (100 µl) to corresponding wells. Controls included a known amantadine-resistant and sensitive virus and a negative control consisting of plain MEM. Plates were centrifuged at 700 x g for 45 minutes at ambient temperature and further incubated at 37°C for 15 minutes. Virus inoculum was removed and replaced with 100 µl MEM (1.25 mg/ml TPCK trypsin and 1.0 µg/ml amantadine where necessary) before the plates were incubated at 37°C with 5% CO_2 for 16 hours. Following incubation, MEM was removed and plates were washed once with PBS. Cells were fixed using industrial methylated spirit followed by washing in PBS (0.01% Tween20; PBST) using a platewasher. Plates were blotted dry and 100 µl mouse monoclonal antibody targeting the viral nucleoprotein (Harlan Sera Labs, Loughborough, UK; cat. no. MAS771b) diluted 1/1000 in PBST (5% w/v fat free milk) was added. Following incubation at 37°C for 60 minutes, plates were washed 4 times using PBST. A secondary sheep anti-mouse IgG antibody conjugated with horseradish peroxidase (HRPO; Sigma Chemical Co; cat. no. A5096) diluted 1/10000 in PBST (5% w/v fat free milk) was added (100 µl) and incubated for 60 minutes at 37°C. Plates were washed 4 times using PBST and blotted dry. 3,3',5'5'-tetramethylbenzidine (TMB; Europa Bioproducts Ltd, Wicken, UK) was added to each well (100 µl) and incubated at room temperature

for 5 minutes in the dark. To stop the reaction, 100 µi 2N H_2SO_4 (Fisher Scientific, Loughborough, UK) was added before the optical density (OD) of individual wells was read using a Multiskan RC plate spectrophotometer (Labsystems) at a dual wavelength of 450 and 620 nm. Dual wavelength measurements were used to reduce the background 'noise' created by scratches or particulate matter on the wells. This ensures a more accurate colorimetric reading of the contents of the wells. Viruses exhibiting less than 50% inhibition in presence of drug were classified as resistant. Inhibition was calculated using Equation 2.2:

EQUATION 2.2 Method for calculating percentage inhibition of influenza when grown in the presence of amantadine

$$\% \text{ Inhibition} = 100 - \frac{\text{OD with drug} - \text{OD control}}{\text{OD without drug} - \text{OD control}} \times 100$$

2.7 Plaque Reduction Assay (PRA)

The plaque reduction assay (PRA) was similar to the virus titration method (section 2.5). To determine the susceptibility of virus replication to amantadine, duplicate sets of plates were set up and quadruplicate wells inoculated with each virus dilution. Two wells were subsequently maintained with amantadine (1.0 µg/ml) and two wells without. Inhibition of the virus to amantadine was determined by counting mean plaque numbers per dilution in replicate wells in the presence and absence of the drug. The percentage reduction in plaque forming units per ml (PFU/ml) was then calculated. Viruses showing greater than 50 percent reduction were considered sensitive and those with less than 50 percent inhibition were resistant.

2.8 Haemagglutination Assay (HA) for Influenza

Using a multichannel pipette, 80 µl PBS was added to the each well of the first row of a 96-well 'U' bottomed plate and 50 µl PBS was added to the remaining seven wells in each of the rows. Culture fluid (20 µl) was added to the first well of the appropriate row, this being repeated for all samples to be tested. The contents of the first row were mixed (five-fold dilution) using a multichannel pipette before 50 µi was transferred to the next well. The mixing and transferring was repeated until the final row (ten-fold to 320-fold dilution) where the last 50 µl fluid was discarded. Fresh turkey erythrocytes were prepared by washing with PBS and a 0.5% suspension was made using PBS as the diluent. Turkey erythrocytes (50 µl; 0.5%) were added to each well and mixed by gently tapping the plate. Plates were incubated at room temperature for 50 minutes and then the haemagglutination titre read by identifying the well exhibiting approximately 50 percent agglutination. This end point was recognised as the point at which there was partial agglutination occurring, recognised as a "doughnut-shaped" agglutination of erythrocytes.

2.9 Haemagglutination Inhibition (HAI) for infiuenza Serology

To analyse serum samples for evidence of previous influenza infection and to determine the nature of the subtype of virus involved in that infection, HAI tests were performed. The patients' sera and control sera were first treated to remove any non-specific inhibitors by using receptor-destroying enzyme (RDE). One volume of serum was added to 4 volumes of RDE and incubated for 18 hours at 37°C. In order to inactivate the RDE, samples were incubated at 56°C for 1 hour. Relevant stock virus strains were chosen according to the requirements for the test. For each virus, a dilution containing 8 agglutinating doses (AD) is prepared and confirmed by back-titrating to 1 AD.

Twenty-five µl of RDE treated serum (1/5 dilution in RDE) was added to the first well of a 96-well 'V' bottom microtitre plate and two-fold serial dilutions prepared across the plates using 25 µl volumes of PBS. The relevant virus (8 AD) was added in 25 µl amounts to every well of the serum titration and plates incubated at room temperature for one hour. Turkey erythrocytes (25 µl; 0.5%) were added to each well and incubated for 30 minutes at room temperature. The test was then

read by tipping the plate so that non-agglutinated cells could be seen running down the bottom of the wells and the HAI titre was read as the last well exhibiting non-agglutination.

2.10 Haemagglutination Inhibition (HAI) for Extended Typing

HA subtype identification of viruses was done by HAI test using a panel of post infection ferret antisera raised to reference virus strains. Ferret antisera were RDE treated and diluted ten-fold to 640-fold in 96-well 'V' bottomed plates as described in section 2.9. The viruses to be analysed were adjusted to 8 HA units and 25 µl amounts added to every well of the serum titration. Following incubation and addition of turkey erythrocytes, plates were read as described in section 2.9.

2.11 Microneutralisation Assay

A microneutralisation assay had been developed and this was used in conjunction with an EIA to test serum samples for neutralising antibody (262, 308). A panel of viruses was constructed including A/Tawian/1/86, A/Texas/36/91, A/Thess/1/95, A/JHB/34/94, A/Shan/9/93 and B/Pan/45/90; viruses were diluted 1 x 10^{-3} in VTM. Serum samples were RDE treated and two-fold serial dilutions prepared in 80 µl volumes in 96-well microtitre plates. The appropriate volume of virus (80 µl) was added to each well, mixed and incubated for one hour at room temperature.

Confluent monolayers of MDCK cells in 96-well plates were washed and 75 µl of virus/serum mixture added to the appropriate well. Plates were centrifuged at 700 x g for 45 minutes, inoculum replaced with MEM (1.25 µg/ml trypsin) and incubated at 37°C with 5% CO_2 for 16 hours.

Upon completion of incubation, wells were washed with PBST and cells fixed using methanol with 2% H_2O_2. Primary mouse monoclonal antibody targeting the viral nucleoprotein (Harlan Sera Labs, Loughborough, UK; cat. no. MAS771b) was diluted in PBS containing 1% BSA (bovine serum albumen) to 1/1000, 100 µl added to each well and incubated at 37°C for 1 hour. Plates were then washed four times with PBST using an automatic platewasher (Labsystems) and blotted

dry. A secondary rabbit anti-mouse antibody conjugated with HRPO (DAKO cat. no. P0260) was made to 1/500 in PBST, 100 µl added to each well and incubated for one hour at 37°C. Plates were washed four times using PBST and wells blotted dry. Substrate was prepared (1.25 ml citric acid (24.3 mM), 2.6 ml Na_2HPO_4 (100 mM), 300 µl AEC (3.3 mg AEC in 1 ml dimethylsulphoxide), 10 µl 30% H_2O_2, and 1.15 ml distilled H_2O) and 50 µl was added to each well and incubated at room temperature for 30 minutes. Plates were washed once with PBST and 100 µl PBS BSA 1% added to each well. Results were read using a light microscope counting the number of visible stained foci per well. End points were calculated by identifying the dilution where 50 percent stained infectious foci were observed when compared to the control wells.

2.12 Polymerase Chain Reaction

2.12.1 RNA Extraction

RNA extractions were performed using a method previously described (33, 86). Virus suspension or tissue culture fluid (150 µl) was added to 840 µl lysis buffer L6 (Severn Biotech Ltd) and 10 µl silica extraction matrix (Severn Biotech Ltd). The mixture was vortexed and incubated at room temperature in a shaker for 10 minutes. The mixture was then centrifuged at 13,500 RPM for 30 seconds and the supernatant carefully discarded. The pellets were washed twice with 1 ml of L2 wash buffer (Severn Biotech Ltd), twice with 1 ml 70 percent ethanol (BDH) and once with 1 ml acetone (BDH). The resulting pellets were dried at 56°C for 10 minutes. Thirty µl nuclease-free H_20 (Promega) and 1 µl Rnasin (40 U/µl; Promega) was added to each pellet, vortexed and incubated at 56°C for 10 minutes to elute the RNA. Each tube was vortexed to resuspend the silica pellet and centrifuged at 13,500 RPM for 5 minutes. Supernatant was carefully removed from the pellet, ensuring no carryover of silica.

2.12.2 Reverse Transcription

A reverse transcription mix (17.8 µl) was prepared containing 4 µl PCR buffer (20 mM), 6 µl $MgCl_2$ (50 mM), 6 µl dNTP (each dNTP 10 mM), 1 µl M-MLV Rtase (200

U/µl; all Gibco BRL), 0.4 µl random hexamer mix (0.53 µg/µl; Pharmacia Biotech), and 0.4 µl RNasIn (40 U/µl; Promega). The mix was added to 22.2 µi of extracted RNA and incubated at room temperature for 10 minutes, then 37°C for at least 45 minutes and 95°C for 5 minutes. The cDNA product was then placed on ice for 5 minutes until required.

2.12.3 PCR Amplification

PCR amplification of the influenza A matrix gene was performed using a single round amplification. Primers (MWG Biotech) used to amplify the coding region were designated AMP2 (forward; 5'-GCAAAAGCAGGTAGATATTG-3') and AMP998 (reverse; 5'-AGGTAGTTTTTTACTCCAGC-3'). A reaction mix containing 8 µl PCR buffer (20 mM), 2 µl $MgCl_2$ (50 mM), 1 µl AMP2 (25 pmol/µl), 1 µl AMP998 (25 pmol/µl), 63.7 µl nuclease free H_2O (Promega), and 0.3 µl *Thermus aquaticus* (*Taq*) polymerase (5 U/µl; Gibco BRL) was prepared for each PCR reaction. The primary mix (80 µl) was added to 20 µi cDNA and amplification performed using a PTC-200 thermocycler (Perkin-Elmer) using the following cycling conditions: 94°C for 2 minutes, then 35 cycles of 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute. Reactions were held at 15°C until required.

Amplification of the HA1 region of the haemagglutinin gene of influenza A H3N2 was performed using a nested PCR consisting of two separate rounds. Primary amplification was performed using primers designated AH3G (forward; 5'-AAGCAGGGGATAATTCTATT-3') and AH3H (reverse; 5'-ATGCCTGAAACCGT-3'). A primary amplification mix consisting of 8 µl PCR buffer (20 mM), 2 µl $MgCl_2$ (50 mM), 1 µl AH3G (25 pmol/µl) and 1 µl AH3H (25 pmol/µl), 63.7 µl nuclease free H_2O (Promega), and 0.3 µl *Taq* polymerase (5 U/µl) was prepared per reaction and 80 µl added to 20 µi cDNA. Amplification was performed using the following cycling conditions: 94°C for 2 minutes, then 35 cycles of 94°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute. PCR products were held at 15°C until required.

Secondary amplification of the HA1 primary PCR product was performed using the primers AH3B (forward; 5'-AGCAAAGCTTTCAGCAACTG-3') and AH3CII (reverse; 5'-GCTTCCATTTGGAGTGATGC-3'). A reaction mix containing 5 µl

PCR buffer, 2.5 µl $MgCl_2$ (50 mM), 1 µl dNTPs (10 mM), 1 µl AH3B (25 pmol/µl) and 1 µl AH3CII (25 pmol/µl), 33.3 µl nuclease free H_2O, and 0.15 µl *Taq* (5 U/µl) per reaction (48 µl) was added to 2 µl of the primary PCR product. Amplification was performed using the following cycling conditions: 94°C for 2 minutes, then 35 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute. PCR products were held at 15°C until required.

2.12.4 Gel Electrophoresis

PCR products for analysis were loaded into a 1% SeaKem agarose gel (FMC Bioproducts) using Orange G loading dye (Severn Biotech). Gel electrophoresis was in 1X tris borate EDTA (TBE) buffer at 80 volts for 90 minutes. Amplicons were visualised by staining with ethidium bromide (10 µg/ml) and viewed using short wave ultraviolet light.

2.13 Sequencing Gel Electrophoresis

Sequencing of the influenza A matrix and haemagglutinin gene was performed using an Applied Biosystems 373 Sequencer. RNA extraction, reverse transcription and PCR amplification were performed as described above (sections 2.12.1 to 2.12.4). The PCR product was loaded into a 1% agarose gel and electrophoresed at 80 volts for 90 minutes. Gels were examined under long wave ultra violet light after staining with ethidium bromide and bands of the predicted size were excised using a clean scalpel.

Two methods were used for the purification of PCR products from agarose gels. Initially, a Gelex DNA Extraction Kit (Camgen) was used; this utilised a silica-based methodology. Gel slices were melted at 56°C with a 0.5 volume of Gelex Modifier and 4.5 volume of Gelex Salt wash buffer. Once the gel had completely melted, 10 µl Gelex Resin (silica) was added and the reaction tube vortexed thoroughly. The reaction was incubated at room temperature for one minute and then centrifuged at 13,500 RPM for 30 seconds. The supernatant was discarded and the pellet washed twice with Wash Buffer. The pellet was dried at 56°C, resuspended in 10 µl of nuclease free H_2O and incubated at room temperature for

1 minute. Following centrifugation, the supernatant was carefully removed and the pellet discarded. To confirm the presence of the desired DNA, a 1 µl sample was electrophoresed on a 1% agarose gel and the remaining eluted DNA was stored at -20°C until required. Because this method proved unsatisfactory for samples containing low yields of DNA, an alternative method was used to ensure that samples containing low yields of DNA could be sequenced.

The QIAquick Gel Extraction Kit (Qiagen) utilised membrane technology (spin columns) and was more reliable than the Gelex DNA Extraction Kit. Gel slices were melted at 56°C in a wash buffer provided, vortexed and loaded into a spin column. The column was centrifuged at 13,500 RPM for 1 minute; the elution waste was then discarded. Further wash buffers were added to the column and eluted by centrifugation. The column was then placed into a clean ependorf tube and 30 µl nuclease free water loaded into the column which was centrifuged again. The eluted DNA was collected and a 1 µl sample was electrophoresed on a 1% agarose gel to ensure that the correct amplicon was present and to estimate the quantity of DNA required for the sequencing reactions. The remaining eluate was stored at –20°C until required.

Sequencing reactions for the matrix gene were prepared using the primers AMP2 (forward; 5'-GCAAAAGCAGGTAGATATTG-3'), AMPB (forward; 5'-CAGAGACTT GAAGATGTCTT-3'), AMPCII (reverse; 5'-TGCTGGGAGTCAGCAATCTG -3'), AMPDII (reverse; 5'-GACCAGCACTGGAGCTAGGA -3'), AMP680 (forward; 5'-ATTGGGACTCATCCTAGCTC -3') and AMP998 (reverse; 5'-AGGTAGTTT TTTACTCCAGC -3'). Sequencing of the influenza HA1 region was performed using the primers AH3G (5'-AAGCAGGGGAAAATAAAAAC-3'), AH3B (5'-AGCAAAGCTTTCAGCAACTG-3'), AH3C (5'-GCTTCCATTTGGAGTGATGC-3') and AH3I (5'-TCCCTCCCAACCATTTTCTA-3'). All sequencing primers were used at a concentration of 3.2 pmol/µl.

Individual reactions were prepared containing 1 µl each primer, 8 µl Ready Reaction mix (Perkin-Elmer), template DNA and nuclease free H_2O. The quantity of DNA added to the reaction was estimated from the intensity of the band observed from electrophoresis of a 1 µl sample on an agarose gel; this usually ranged from 5 µl to 8 µl. The total volume of the reaction was made up to 20 µl with nuclease free H_2O. Sequencing reactions were subjected to the following

cycling conditions: 25 cycles of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes. Products were then held at 4°C.

Upon completion of cycling, products were purified immediately to avoid template degradation. The entire sequence product to be purified was added to a mix of 50 µi cold ethanol (95-100%; BDH) and 2µl sodium acetate (3 M, pH4.6), cooled on ice for 10 minutes and then centrifuged at 13,500 RPM for 20 minutes. The supernatant was discarded and replaced with 250 µl cold ethanol (70%) and spun at maximum speed for 5 minutes. The supernatant was removed using a fine-tipped Pasteur pipette and pellets dried using a DNA Speed Vac (Savant, France) for 5 minutes. Pellets were stored at -20°C until further required for sequencing.

Sequencing was performed by the dideoxy chain termination method (315). The reactions were analysed by separation on polyacrylamide-urea gels using a 373 sequencing machine (Applied Biosystems).

2.14 Sequence Analysis and Construction of Phylogenetic Trees

Output files from the 373A sequencer were edited in the program Analysis (Applied Biosystems) to remove waste sequence from the start and end of each sequence. Files were then transferred to SeqEd (Applied Biosystems) where the forward and reverse sequences were compared and aligned. The consensus sequence was converted to a text format and transferred to EditSeq (DNAStar). The EditSeq files were imported into MegAlign (DNAStar) and aligned using the Clustal algorithm option.

Phylogenetic trees were constructed using the PHYLIP shareware package on a Power Macintosh. Initially, alignments from MegAlign were modified to a compatible layout. The alignment files were exported in Paup format and converted into PHYLIP format using the program Readseq. This file format was then used in other programs within the PHYLIP package. Transition:transversion ratios (Ts:Tv) were calculated using Tree Puzzle 4.0. Maximum likelihood distance matrices were calculated using the program DNADist (PHYLIP). The output file was then analysed by Fitch, which produced a tree in text format. The tree was viewed using the program Treeview.

To determine the strength of nodes and branches within each tree, bootstrap values were calculated. Seqboot was used to create 100 datasets, the output file was then imported to DNADist, which calculated the maximum likelihood distance matrices for each dataset. The output file from DNADist was imported to Fitch where trees were produced for each dataset. Consence was used to produce the consensus or majority tree with bootstrap values on each branch. Trees and bootstrap values were viewed in Treeview. Completed phylogenetic trees were created by transposing bootstrap values onto the original trees. Values greater than 70 were displayed although where nodes were considered of phylogenetic importance, bootstrap values were included irrespective of value.

2.15 Cloning

2.15.1 Transformation of Competent Cells

Cloning of the HA1 gene of influenza A was performed using a TOPO TA Cloning Kit (Invitrogen). Briefly, a vial of competent *Escherichia coli* (*E. coli*) cells was thawed on ice. To each vial 2 µl β-mercaptoethanol was added and gently mixed. Two µl of a cloning reaction composed of 1 µl pCR-TOPO vector (Invitrogen), 2 µl PCR product and 2 µl sterile nuclease free H_2O was added to the competent cells and incubated on ice for 30 minutes. The cells were heat-shocked for 30 seconds at 42°C and then incubated on ice for two minutes. SOC medium (250 µl; Invitrogen) was added and the cell suspension incubated in an orbital shaker (Gallenkamp) for 30 minutes at 37°C and 180 RPM. Agar plates were poured containing ampicillin sodium salt (20 mg/ml; Life Technologies) and allowed to cool. The plates were spread with 40 µl X-gal (40 mg/ml) and 40 µl IPTG (100mM) and incubated at 37°C for approximately one hour prior to use. One hundred µl of transformed cells was spread onto the plates which were incubated at 37°C overnight.

2.15.2 Isolation of Colonies Containing PCR Product of Correct Size

Approximately 10 to 20 white colonies were picked using a sterile loop and spread onto a fresh agar plate (as above) and incubated at 37°C overnight. Ten new colonies were picked and the loop from each colony was immersed into a vial containing 5 ml broth; this was then incubated in a shaker at 37°C overnight. Each loop was also immersed into a vial containing 50 µl sterile H_2O, which was then placed into a boiling water bath for 5 minutes. The vial was centrifuged at 13,500 RPM for 1 minute and the supernatant carefully removed. A PCR mix containing 5 µl PCR buffer (20 mM), 3 µl $MgCl_2$ (50 mM), 1 µl dNTP (each dNTP 10 mM), 1 µl M13 forward primer (20 pmol/µl; 5'- CAGGAAACAGCTATGAC-3'), 1 µl M13 reverse primer (20 pmol/µl; 5'- GTAAAACGACGGCCAG-3'), 28.8 µl sterile H_2O and 0.25 µl *Taq* (5 U/µl) was added to 10 µl supernatant (total volume 50 µl) and amplified using the following cycling conditions: 29 cycles of 94° for 1 min, 55°C for 1 min, 72°C for 2 min and then 72°C for 5 min. Reactions were then held at 15°C until required. Products were electrophoresed on a 1% agarose gel to identify bands of the correct size. The corresponding broths to these positive results were saved.

2.15.3 Sequencing of Cloned HA1 Gene from Positive Colonies

To sequence the clones, 5 µl of broth was added to 50 µl sterile H_2O and the above selection procedure carried out. The entire PCR product was run on a 1 % agarose gel and the corresponding band excised. Gel slices were cleaned and the DNA extracted using the QIAquick Gel Extraction method (section 2.13). Sequencing primers were identical to those used in routine sequencing of the HA1 gene. The M13 reverse primer (as above) was included (3.2 pmol/µl) in the sequencing reaction to ensure that the whole HA1 region was sequenced.

2.16 Epidemiological Analysis

Data collected by the Birmingham Unit of the Royal College of General Practitioners (RCGP) on the rates of influenza and influenza-like illness was presented as the number of new episodes of illness occurring per 100,000 population (87). This set of data represents an accurate picture of the circulation of influenza activity within the community and was used to plot the incidence of influenza activity in the UK over the past 30 years. Isolates prior to 1995 were predominantly from hospital laboratories and hospitalised cases of influenza. Isolates post-1995 included a greater proportion (approximately 50%) of community-based specimens from general practitioners and virological surveillance studies. Isolates were received from sites all over the UK and therefore represented the circulation of influenza throughout the UK population in all seasons studied.

Any epidemiological or clinical data for viruses was extracted from national archive information consisting largely of laboratory records, EXCEL databases and where possible original patient forms. Details that were collected included the origin of each virus isolated, age and sex of patient and whether amantadine had been used to treat the infection.

Chapter 3

Amantadine Susceptibility Screening of Influenza A Isolates from Clinical Samples in the United Kingdom (1958-1999)

3.1 Introduction

3.1.1 Use of Amantadine

Until the recent launch of the neuraminidase inhibitors zanamivir and oseltamivir, the anti-M2 protein drug amantadine and its analogue derivative rimantadine were the only antiviral drugs licensed for the treatment of influenza infections. For over three decades they have been proven clinically useful in the treatment and prevention of influenza A (37, 74, 149, 165). Amantadine has been available for clinical use since 1966 but has been used only on a limited scale in some countries, especially the UK (277). The use of amantadine over the past three decades has not yet resulted in the appearance of epidemic strains of influenza A virus that are resistant to the compound (144). However, the circulation of resistance strains has been documented illustrating the need for further surveillance (156, 277, 417).

3.1.2 Previous Susceptibility Studies

3.1.2.1 Treatment Groups

Numerous studies have demonstrated how amantadine-resistant viruses are readily selected in animals and tissue culture when virus is grown in the presence of drug (10, 22, 51, 277, 350). From its early days, this was acknowledged as the main disadvantage of the drug but the significance of generation of resistance *in vivo* was not clear. There have been several previous studies to investigate the frequency and occurrence of amantadine-resistant viruses in clinical isolates. Drug-resistant viruses have been recovered from patients undergoing treatment with amantadine or rimantadine (130), patient-contacts (147) and in non-treated persons (156, 166). The groups where an increase in drug-resistant viruses is observed are from patients undergoing amantadine or rimantadine treatment or prophylaxis where selective pressure on the virus was greatest. The frequency of the recovery of these isolates has been determined in several cases (Table 3.1).

TABLE 3.1 Frequency of resistance and documented transmission of amantadine- and rimantadine-resistant viruses from treatment/prophylaxis-based susceptibility studies

Study	Year	Subject Group	Resistance	Transmission	Reference
Hall *et al.*	1987	Children	27%	✗	(130)
Hayden *et al.*	1989	Families	29%	✓	(147)
Hayden *et al.*	1991	Adults	50%	✗	(152)
Mast *et al.*	1991	Elderly	60%	✓	(238)
Houck *et al.*	1995	Elderly	50%	✗	(166)
Gravenstein *et al.*	2000	Elderly	33%	✗	(119)

A study by Hall *et al.* (1987) to investigate the efficacy and acceptability of rimantadine in the treatment of children with acute influenza A infections observed that of 37 children treated with drug, 27% were shown to shed resistant isolates and 45% of children who had received rimantadine were still shedding resistant virus on day 7 after treatment (130). This work provided good evidence that under the influence of drug therapy, resistant viruses were frequently isolated from children and were apparently shed for relatively long periods after treatment had ceased. Hayden *et al.* (1989) investigated the efficiency of rimantadine in protecting family members from influenza A infection and the emergence of resistant viruses during therapy (147). The study found 29% of adults or children undergoing therapy shed resistant virus on day 5 of treatment. It was concluded that rimantadine was ineffective in protecting household members from influenza A infection due to the close proximity of patients. When rimantadine was used for both treatment and post-exposure prophylaxis, resistant viruses were rapidly selected and transmitted between family members. From other similar studies, it can be concluded that amantadine- and rimantadine-resistant viruses have been recovered from 30-60% of adults and children treated for influenza A infections. The findings from Hall *et al.* (1987) and Hayden *et al.* (1989) had important implications in patient-care management (130, 147). The apparent shedding of resistant viruses after the completion of treatment and the transmission of resistant viruses between patients and contacts demonstrated that in situations where patients were in close proximity there was a probability that disease could spread

rapidly. This was especially important in respect to 'at-risk' groups where the acquisition of a resistant influenza A virus could be life-threatening.

When compared with neuraminidase inhibitors, the limiting factors associated with anti-M2 protein inhibitors are highlighted. An investigation into seasonal prophylaxis of nursing home residents compared the efficacy of zanamivir and rimantadine at preventing disease (119). Analysis of viruses shed from patients revealed that in the rimantadine-treated group, 38% of viruses isolated were resistant and 44% of cases from this group were drug-prophylaxis failures. Within the zanamivir-treated group there was no resistance detected and efficacy was improved (119). It is apparent that the emergence of amantadine- and rimantadine-resistant viruses can cause the failure of drug treatment and prophylaxis. The apparent transmission of resistant viruses can result in illness among household contacts and nursing home residents (147, 238). The resistant viruses appear to emerge rapidly and cause typical influenza in human contacts suggesting that wild-type sensitive strains exhibit no selective advantage over resistant strains.

Other important findings from the study of resistant viruses have helped in understanding their properties so that preventative measures might be initiated. The rapid emergence of resistant viruses has been documented; in the avian model this can be from 2-9 days (22, 384) and in humans is typically 4-5 days post-treatment (130, 147). It is apparent that once drug-resistant virus has emerged from a subject, the genotype of subsequent isolates remains resistant or comprises a mix of resistant and sensitive viruses (22).

3.1.2.2 Populations

The emergence of amantadine-resistant viruses in hosts undergoing drug therapy provides valuable data for improving patient care. However, it is equally important to understand the occurrence of amantadine-resistant viruses within the general population, where the use of amantadine is significantly lower. This type of research has been less frequent but several groups have documented the occurrence of amantadine- and rimantadine-resistant viruses in larger samples of

virus isolates (Table 3.2). An early study by Heider *et al.* (1981) tested strains that had been collected from the 1980 Berlin epidemic; 2/21 strains of H3N2 virus were resistant to amantadine and rimantadine (156). The exact origin of these resistant strains was unknown but anti-influenza treatment had not been practised in the former GDR at that time and therefore it was concluded that the strains had been imported from countries where use of the drug had been utilised for several years, e.g. the former USSR (418). This provided evidence for the first time that resistant strains circulated in nature (156). In the former USSR, where rimantadine treatment had been in long-term use since 1969, Kuber *et al.* (1989) noted differences in resistance between different subtypes of influenza A viruses (196). The percentage of drug-resistant variants isolated was 4.7% for H1N1 and 40.1% for H3N2. These figures might have been influenced by the proportion of subtypes circulating in the population i.e. H3N2 subtypes were predominantly circulating the population, but they also might have indicated differing sensitivities between different subtypes. The study by Kubar *et al.* (1989) concluded that despite the prolonged use of rimantadine (two decades) the efficacy of the drug was undiminished (196).

TABLE 3.2 Frequency of resistance of amantadine- and rimantadine-resistant viruses from population-based amantadine/rimantadine susceptibility studies

Study	Date	N*	Study Period	Resistance	Reference
Heider *et al.*	1981	21	1980	9%	(156)
Pemberton *et al.*	1983	39	1978-1981	15%	(277)
Belshe *et al.*	1988	248	1978-1988	2%	(23)
Valette *et al.*	1993	105	1988-1990	0%	(364)
Prud'homme *et al.*	1997	50	1991-1994	0%	(287)
Ziegler *et al.*	1999	2017	1991-1995	0.8%	(417)
Elliot & Zambon	2001	2309	1958-1999	2.2%	(85)

***Number of patients studied.**

Other previous studies screening clinical isolates for amantadine-resistance have been limited. Two of the most significant were studies by Belshe *et al.* (1989) and Zeigler *et al.* (1999). Belshe's group screened 248 viruses from clinical isolates originating in the US over a period of ten years (1978-1988). Of these, 5 (2%) were resistant to amantadine and rimantadine (23). However, the resistant viruses were all isolated from members of one family undergoing rimantadine treatment and therefore did not represent a true figure of resistance within the population. This study demonstrated the absence of naturally-occurring resistant viruses and concluded that the emergence of new influenza strains may have prevented the widespread emergence of resistant influenza A virus. The most recent susceptibility screening study was by Zeigler *et al.* (1999) who tested isolates from 43 different countries around the world (417). Resistant viruses were detected at a frequency of 0.8%. Of the resistant viruses found, 12/16 were isolated from cases where there had been no apparent exposure to amantadine or rimantadine (417). This study concluded that although the circulation of resistant strains was rare, there was evidence to suggest that resistant strains were emerging without the selective pressure of drug. Studies by Prud'homme *et al.* (1997) and Valette *et al.* (1993) screened 50 Canadian isolates from 1991-1994 and 105 French isolates from 1988-1990 respectively using an EIA similar to the one described for this study (287, 364). Data resulting from this work demonstrated that all isolates were sensitive to amantadine, further evidence for the low frequency of naturally occurring resistance.

Accumulated data from previous studies shows the importance of continuing surveillance for naturally-occurring influenza A viruses, especially in the advent of widespread use of antiviral compounds. In untreated patients it has been reported that approximately 0.8% of clinical isolates recovered from patients are resistant (144). The predominance of resistant strains in drug-treated patients and the low frequency of naturally-occurring drug-resistant strains suggests that drug-resistance does not confer any selective advantage.

3.1.3 Transmission of Resistant Viruses

In the avian model there have been studies that have demonstrated the ability of amantadine-resistant viruses to be transmitted between birds. Webster *et al.* (1985) studied the control of a highly pathogenic influenza virus among chickens (384). Infected chickens were administered with amantadine or rimantadine both prophylactically and by treatment. The drugs were found to be effective in the prevention of disease but did not stop virus shedding. In an attempt to mimic natural conditions, infected birds were put in contact with susceptible birds and all were administered amantadine. It was found that the susceptible birds all succumbed to disease, direct evidence for the transmission of resistant virus and its potential for causing disease (384). Again, using the avian model, Bean *et al.* (1989) infected chickens with virus and treated the birds with amantadine (22). After exposure to drug, the infected birds shed resistant virus and continued to shed after treatment had stopped. When birds carrying resistant virus were mixed with birds infected with wild-type sensitive virus for which no amantadine treatment had been administered, resistant virus was isolated from the sensitive birds within one passage. This further demonstrated the potential for transmission of resistant viruses between close contacts in animal populations (22).

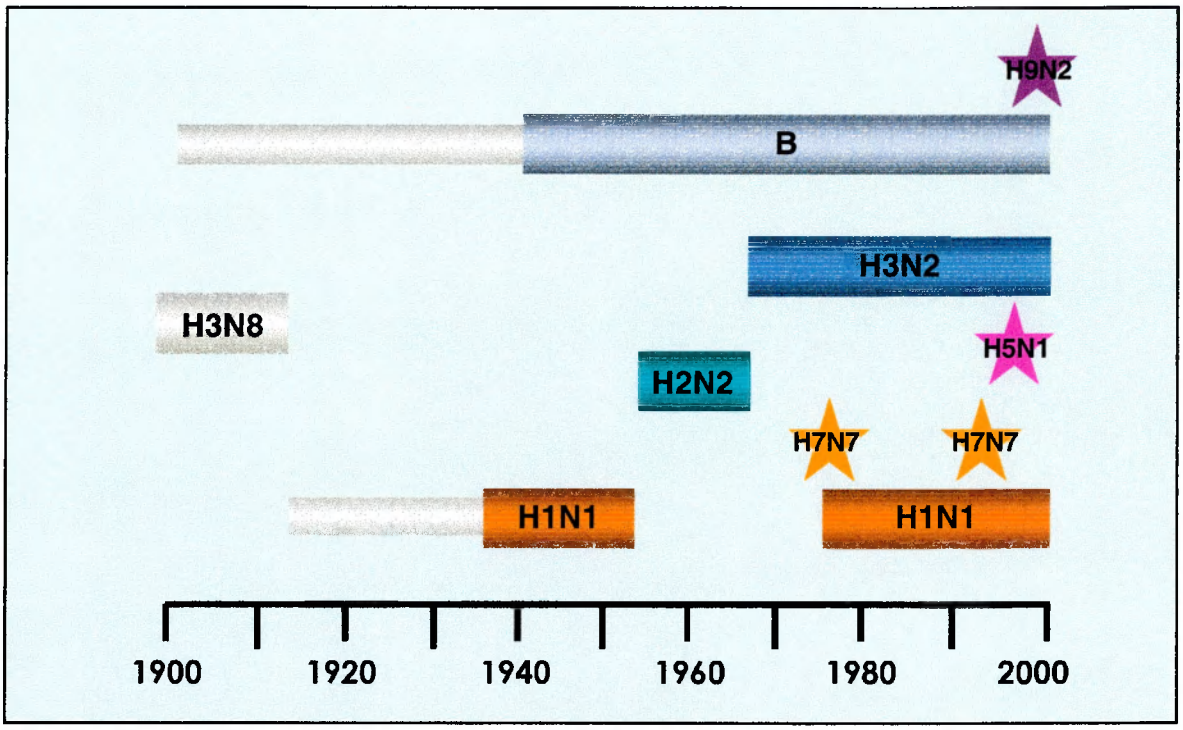
Transmission of resistant viruses in human populations has been limited. Some evidence to support the case for transmission was produced by Hayden *et al.* (1989) where the emergence of resistant viruses among family members treated with amantadine was studied (147). Within families treated with amantadine, illness in contacts was apparently caused by the transmission of a resistant virus from the index case (147). Another study providing evidence for the transmission of human resistant viruses was Mast *et al.* (1991) who investigated outbreaks of influenza A disease in nursing homes (238). Within the homes, patients contracting influenza were treated with amantadine two days after the onset of symptoms and general amantadine prophylaxis was initiated shortly after the onset of the outbreak. Resistant viruses were isolated from several patients. The viruses isolated had identical genetic changes and the patients were living in adjacent rooms providing further evidence for the transmission of resistant viruses in humans (238).

Population studies of amantadine susceptibility have not yielded any documented cases of the transmission of resistant viruses without the selective pressure of the antiviral drug. This suggests that resistant viruses emerging in nature do not have any obvious selective advantage over sensitive viruses that allow them to be transmitted. This theory alone may be partly responsible for the absence of a circulating epidemic human influenza strain that is resistant to amantadine or rimantadine.

3.1.4 Viruses to be Screened (UK 1958-1999)

Over the last century there have been several different influenza A subtypes that have circulated the globe (Figure 3.1). Evidence for circulation of certain subtypes from the earliest periods of the last century has been gathered from serological evidence. This data has revealed that H1N1 viruses emerged in approximately 1918 and circulated until 1958. The first isolation of influenza viruses occurred in the early 1930s (335) and from this point in history there is a relatively accurate record of the viruses and subtypes in circulation. H2N2 viruses superseded H1N1 viruses in 1958 and caused the worldwide pandemic of Asian influenza (81). The circulation of H2N2 viruses spanned approximately ten years after which they were replaced in circulation by another newly emerging subtype. The winter of 1969 saw the emergence of H3N2 viruses, a novel subtype that heralded another worldwide pandemic (Hong Kong influenza) (52). H3N2 remained the only circulating subtype in the UK until 1977 when viruses of H1N1 subtype re-emerged (197). Over the past decade, there have been no major epidemics observed within the UK and currently influenza B, and influenza A H3N2 and H1N1 co-circulate.

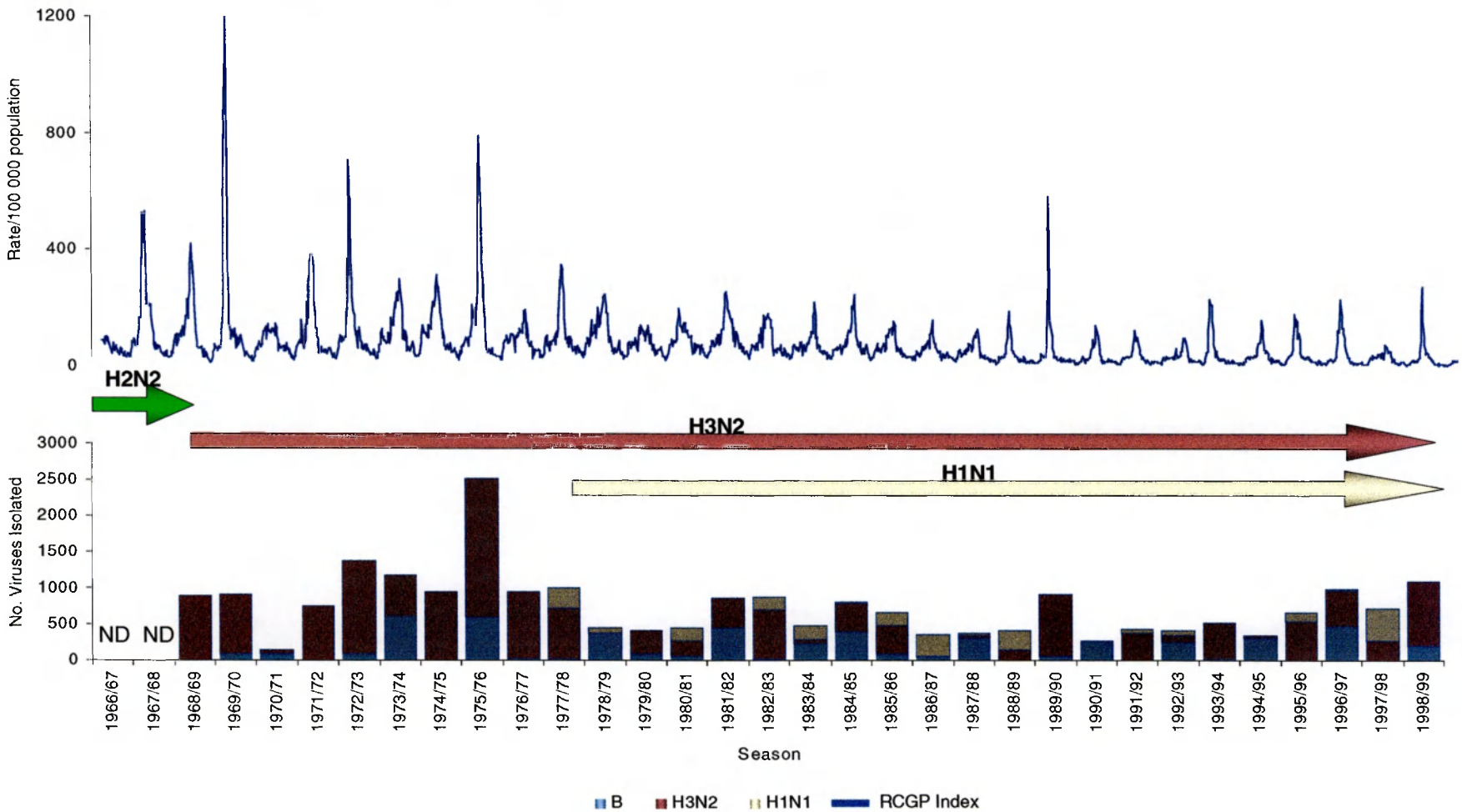
FIGURE 3.1 Circulation and recycling of influenza throughout the last century



Sporadic cases of animal to human transmission of various influenza A subtypes are highlighted in stars. Bold boxes are present where there is direct evidence i.e. virus isolation of circulating subtypes; white boxes represents subtypes where serological data provided evidence for the circulation of virus. Adapted from (342).

The circulation of influenza over 40 years (1958-1999) in the UK is summarised in Figure 3.2. This illustrates the viruses that were circulating the UK over the study period and is very similar to the worldwide pattern of circulation of subtypes. The earliest subtype of influenza A available to be screened were H2N2 viruses. The actual number of viruses screened from this period was low due to poor archiving at the time of isolation and therefore did not represent proportional sampling. Within the UK, H3N2 viruses emerged in the season of 1968/69 causing widespread infection throughout the population. Evidence for the effect of this wave of disease on the UK population can be seen in the Royal College of General Practitioners (RCGP) index where there is a large peak in the rate of General Practitioner (GP) consultations per 100,000 population (Figure 3.2). H3N2 and H1N1 co-circulated the UK with influenza B viruses although the isolation rate for H3N2 viruses was predominant in most seasons. Other virological events of interest within the UK include two large epidemics during the seasons of 1975/76 and 1989/90 that can be identified by peaks in the RCGP index.

FIGURE 3.2 Laboratory confirmed cases of influenza A in the UK



Coloured arrows represent the predominant circulating subtypes and the RCGP index is included to indicate numbers of GP consultations per 100,000 population for influenza-like-illness. No virus isolation data or RCGP index was available for influenza seasons pre-1966/67, therefore the influenza seasons 1958/59 to 1965/66 have been omitted from the chart. ND – no data.

3.1.5 Antiviral Susceptibility Assays

Several methods used to assess the susceptibility of influenza to antiviral drugs have been developed over the years. Hamzawi *et al.* (1981) used an egg-bit assay to culture viruses in the presence and absence of drug (132). Influenza viruses were inoculated onto allantois-on-shell cultures in varying concentrations and incubated: haemagglutination was used to detect antigen (132). Heider *et al.* (1981) assayed the susceptibility of strains from the 1980 Berlin epidemic using a haemadsorption reduction test in mouse Ehrlich ascites cells (156). These two early methods were superseded by the plaque reduction assay that was developed by Appleyard (1975) to study inhibitors of influenza viruses (11). This assay was then commonly used in several susceptibility studies and is still widely used and considered to be the "gold standard" for antiviral susceptibility testing of influenza (31, 148, 242). The plaque assay has been used for antiviral drug testing of other viruses including herpes simplex virus (355), human immunodeficiency virus (295) and cytomegalovirus (386).

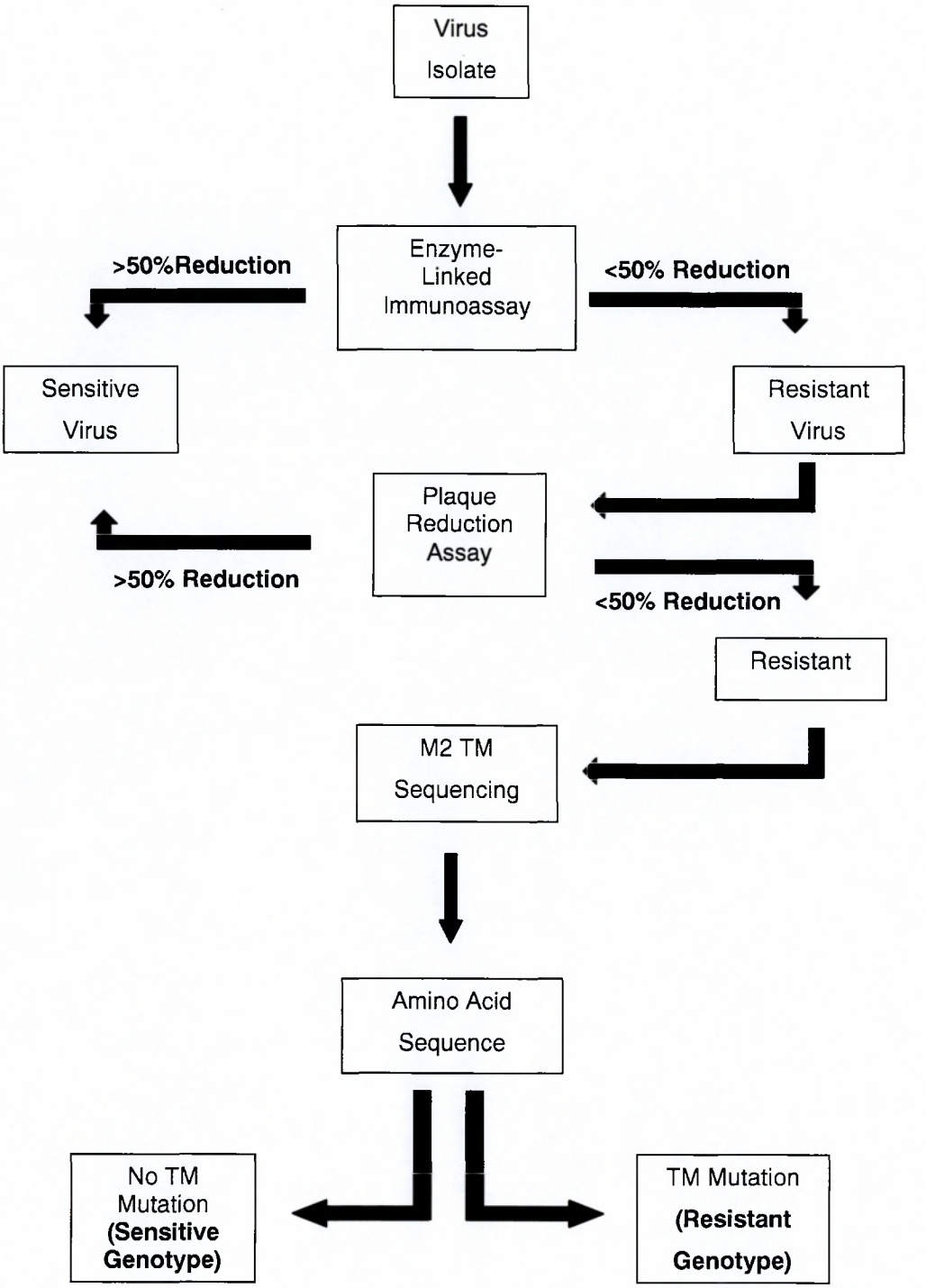
The enzyme-linked immunoassay (EIA) was first utilised by Belshe *et al.* (1989) to screen viruses for amantadine susceptibility (23). The assay that the group developed has been modified and used in several other studies including the present one demonstrating its usefulness for such work (256, 287, 364, 417). Molecular biology has allowed the development of methods that can be used either as screening tools or to directly analyse the amino acid sequence of viruses. Polymerase chain reaction-restriction analysis has been used to detect mutations within M2 known to confer resistance (193). This has been used as an alternative to the plaque reduction assay to confirm resistance detected by an EIA (417) but the method can only detect viruses carrying mutations at positions previously reported to confer resistance, any viruses with changes outside of these positions would not be detected. Direct automated sequencing has been used as a tool to screen viruses for amantadine-resistance but is more commonly used to determine the sequence of the M2 gene and identify mutations arising.

To enable the collection of data within the current study, an antiviral susceptibility screening protocol had to be established. The options available for determining amantadine susceptibility of viruses were tissue culture (plaque reduction assay), EIA or molecular techniques e.g. polymerase chain reaction-restriction analysis.

The assay was to be used as a tool to screen viruses collected and archived from the UK over the past 40 years, therefore it was essential that it was quick, with relatively low labour input and with the capability to screen large numbers of isolates. Although considered the gold standard for antiviral susceptibility testing, using the plaque reduction assay to screen isolates was unrealistic because it was extremely labour intensive resulting in a low throughput of isolates; polymerase chain reaction-restriction analysis was also considered too labour intensive. Therefore, it was decided that an EIA would be used initially to screen the large number of virus isolates and any resistant viruses detected would be confirmed resistant and further characterised by plaque reduction assay. These resistant viruses would then be genotypically characterised by sequencing the M2 gene to determine the drug genotype (Figure 3.3).

The plaque reduction assay has been described as the gold standard for assessing the susceptibility of influenza to antiviral compounds (409) and has been used with amantadine for many years (11, 148). The advantage of the plaque assay over the EIA is that growth of the virus is calculated as individual infectious particles or plaque forming units (PFU). It is considered to have greater accuracy in determining the infectivity of a virus, compared to EIA. A recent study that outlined the optimisation of a cellular EIA for screening antiviral agents found that results from the EIA correlated strongly with the plaque reduction assay (256). However, even with comparable susceptibility data, the plaque reduction assay has the advantage that valuable data concerning the phenotypic characteristics of a virus can be determined through studying plaques produced from infection. These data include the general size and number of plaques, and the morphological characteristics of individual plaques. This can provide evidence for the growth potential of the viruses tested and for the presence of viral quasispecies within the isolate.

FIGURE 3.3 Antiviral susceptibility screening protocol



Flowchart illustrates the antiviral susceptibility testing protocol used in the determination of frequency of amantadine-resistance in this study.

3.2 Aims

The aim of this work was to assess the frequency and pattern of occurrence of amantadine-resistance in influenza viruses circulating within the UK. Amantadine-resistance within the UK population has not previously been studied; as a consequence of the low use of the drug within the population, results should indicate the level of naturally-occurring resistance within circulating strains. With access to an archive of isolates collected over the past 50 years it was possible to screen a large number of viruses including periods when amantadine was not in use. An assay was designed and optimised which enabled efficient collection of data. The biological and phenotypic properties of resistant isolates were characterised by plaque reduction assay that provided data on growth and resistance characteristics. From collected data, several questions were posed:

- What was the frequency of amantadine-resistance in the UK through the period 1958-1999?
- Is there variation in amantadine susceptibility between influenza different antigenic subtypes?
- Was there evidence that susceptibility varied between influenza seasons?
- Did the transmission of resistant viruses occur within the population?
- What was the potential for the emergence of an epidemic strain resistant to amantadine?

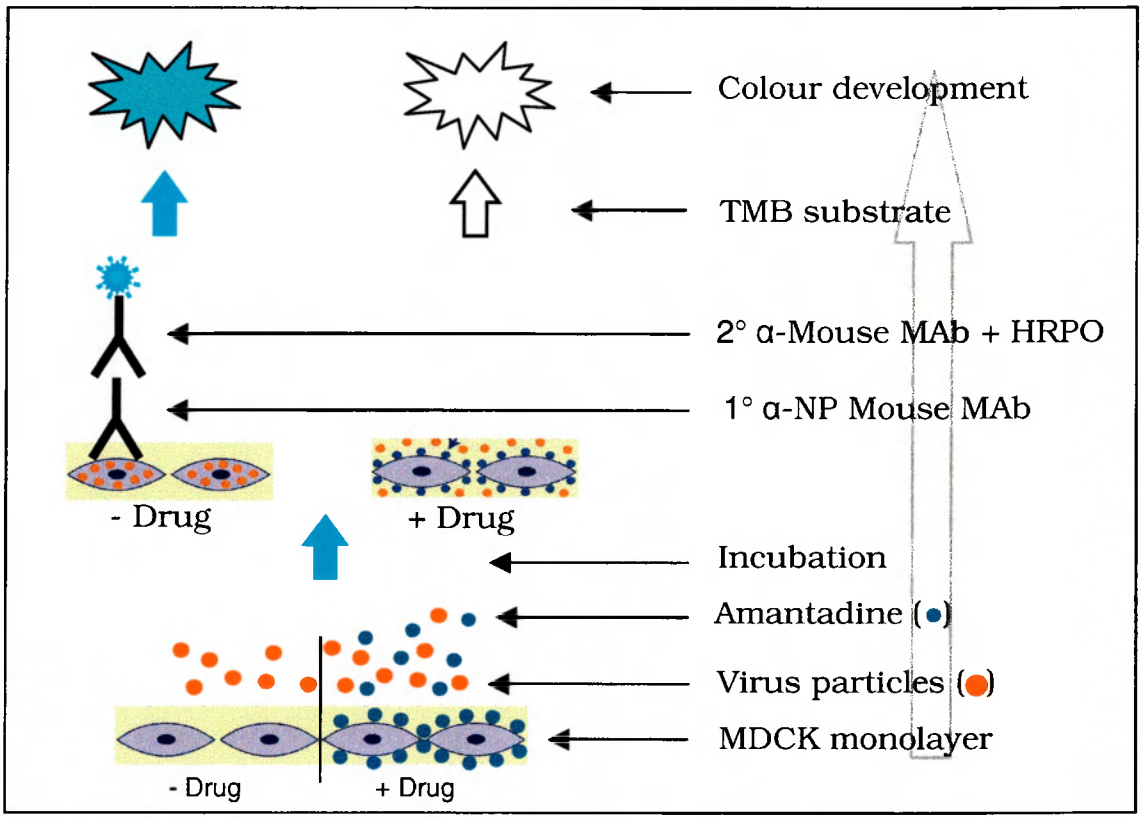
3.3 Results

3.3.1 Design of an Enzyme-Linked Immunoassay (EIA)

The final protocol for the susceptibility EIA is described in Chapter 2 (section 2.6; page 41) but during the development and optimisation of the assay several important parameters were assessed including the optimisation of both primary and secondary monoclonal antibody (MAb) concentrations, the incubation times for Mab and substrate, and the composition of antibody diluents. The incubation time for propagating viruses prior to antibody staining was an important consideration. The length of time would determine whether single cycle or multiple cycle replication had occurred. Results demonstrated that following an incubation period allowing single cycle replication only the strength of signal produced (representing antigen released) was weak and would not permit the detection of viruses with low infectivity titres. Signal produced after a multiple cycle incubation of 16 hours was significantly stronger and allowed detection of viruses with both low and high titres.

Finally, controls for the assay had to be included to validate results. A reference virus strain (A/Tawain/1/86) was used for the amantadine-sensitive control and a known amantadine-resistant virus (GS10) used as the resistant control. To monitor background negative values, cells were infected with sterile virus transport medium (+ / - drug) and stained as normal to highlight changes within the quality of the cell monolayer or toxicity to the drug. A set of controls was included with each batch of assays performed and the susceptibility of the sensitive and resistant controls monitored. If either control appeared to have failed validation criteria (i.e. resistant control demonstrating >50% inhibition) the results were discarded and the assay repeated. The EIA susceptibility assay is summarised in Figure 3.4.

FIGURE 3.4 Diagrammatic representation of the EIA-antiviral susceptibility assay



Cells were infected with virus and incubated in the presence and absence of amantadine. Two monoclonal antibodies were used to detect the presence of viral nucleoprotein. The level of protein expression was measured colourimetrically using HRPO and TMB.

3.3.2 Frequency of Amantadine-Resistance in the UK 1958-1999

The viruses used in this study were all influenza A isolates that had been characterised at the Influenza Laboratory based at the Central Public Health Laboratory (CPHL), Colindale, which is the national influenza laboratory. A range of specimens consisting mainly of throat and nose swabs and tissue culture fluid was sent to the laboratory from regional public health laboratories, hospital laboratories and general practitioners. Viruses had been predominantly isolated in mammalian tissue culture (>75%) while viruses isolated prior to 1985 had been isolated in both the allantoic cavity of fertile hens eggs and tissue culture. Viruses were stored at –70°C or freeze-dried at time of isolation. The origin of isolates prior to 1995 was predominantly from hospital laboratories and hospitalised cases of influenza. Isolates following this date included a greater proportion (approximately 50%) of community-based specimens from general practitioners and virological

surveillance studies. Isolates were received from sites all over the UK and therefore represented the circulation of influenza throughout the UK population in all seasons studied.

A total of 2309 influenza A isolates were tested using the susceptibility EIA. Of the viruses tested, positive signal was detected in 2130 (92.2%). Viruses that did not grow sufficiently for analysis were generally not used, except in seasons when the number of archived viruses was low, isolates failing to produce signal were inoculated into MDCK cells. After incubation for 7 days at 37°C in an attempt to amplify antigen, antiviral screening using the EIA was repeated; if insufficient signal was still produced the virus was discarded. The number of viruses archived limited the proportion of subtypes available for testing but the number of H3N2 and H1N1 tested represented an approximation of the predominant circulating strains over the last 40 years. In total, positive signal was detected for 1559 (72.5%) H3N2, 531 (25.6%) H1N1 and 40 (1.9%) H2N2 viruses. Viruses were categorised into defined seasons depending on their date of isolation to enable data to be compared between each influenza season. Officially, for UK surveillance purposes, the influenza-monitoring season starts in the UK on October 1st and lasts until March 31st although the actual duration of influenza epidemics is probably 8-12 weeks between these dates (98). Approximately 95% of viruses used in this study were isolated during the time of influenza epidemics each year, the remainder were isolated from sporadic cases and outbreaks occurring in months when there was very limited influenza circulation in the country. Viruses that were isolated out of season were assessed according to the date of isolation and their antigenicity and grouped into an appropriate season.

Viruses demonstrating less than 50% inhibition to amantadine were classified as drug-resistant and collected for further analysis. Initial results revealed that the susceptibility EIA had detected resistant viruses in H3N2 and H1N1 subtypes but the small group of H2N2 viruses tested lacked any resistance. The frequency of detection of resistant viruses by EIA for H3N2 and H1N1 was 17.8% and 15.8% respectively. When these resistant viruses were further tested using plaque reduction assay it was found that only a small number were confirmed resistant. The subsequent frequency of resistance for H3N2 and H1N1 was 2.4% and 2.1% respectively. The overall frequency of amantadine-resistance between H3N2, H1N1 and H2N2 viruses (1958-1999) was 2.3% (Table 3.3).

TABLE 3.3 Summary of influenza A susceptibility screening

Subtype	Circulation (years)	N	Resistant by EIA	Resistant by Plaque Assay
H3N2	1968-present	1559	278 (17.8%)	37 (2.4%)
H1N1	1977-present	531	87 (15.8%)	11 (2.1%)
H2N2	1958-1968	40	0	0
TOTAL	**1958-present**	**2130**	**365 (17.1%)**	**48 (2.3%)**

N – number of viruses tested.

When seasonal data were analysed, the results indicated that the occurrence of resistance was not constant from season to season. There appeared to be 'bursts' of resistance present in some seasons and very low levels of resistance in others. This phenomenon was observed in both H3N2 and H1N1 viruses. The pattern of varying resistance by EIA within the H3N2 viruses (Table 3.4) was extremely pronounced. Of 27 seasons screened, 8 contained proportionally larger numbers of resistant viruses to other seasons. Between these peaks of high frequency resistance there were interspersed periods where no resistant viruses had been isolated. This phenomenon was demonstrated when data from 1982/83 to 1985/86 was analysed. There were 25 resistant viruses detected by EIA during the 1982/83 season followed by two seasons where there was a low occurrence of resistance; however, the next season (1985/86) produced 50 resistant viruses, the highest detected for any season. Examining resistance from plaque reduction data, this trend was less pronounced but the season of 1985/86 remained as containing a significantly higher number of resistant viruses than any other season. Data from H1N1 subtypes was similarly analysed and it was again found that several seasons contained proportionally higher numbers of resistant viruses by EIA and were interspersed with seasons lacking any detectable resistance (Table 3.5). For example, 18 resistant viruses were found by EIA in the 1983/84 season but during the following two seasons no resistance was detected. Then, in the 1986/87, 1987/88 and 1988/89 seasons 24, 8 and 9 resistant viruses were isolated respectively. This again demonstrated the sporadic bursts of resistance appearing followed or interspersed with periods of relatively high sensitivity in viruses isolated. Data from plaque reduction assay revealed the 1983/84 season as

containing the highest number of resistant H1N1 viruses. The susceptibility screening of H2N2 viruses revealed a total lack of resistance (Table 3.6).

TABLE 3.4 Amantadine susceptibility screening data for influenza A H3N2 viruses

Season	No. Isolated [1]	No. Tested [2]	No. Tested Virus Analysed (%) [3]	No. Resistant by EIA	No. Resistant by Plaque Assay
1999/98	886	96	76 (79)	19	2
1998/97	453	52	43 (83)	3	1
1997/96	504	251	251 (100)	16	1
1996/95	532	3	3 (100)	1	0
1995/94	40	39	39 (100)	1	0
1994/93	515	148	111 (75)	6	1
1993/92	86	45	33 (73)	3	0
1992/91	367	260	223 (86)	27	2
1991/90	0	0	0	0	0
1990/89	849	169	130 (77)	24	1
1989/88	145	75	58 (77)	10	1
1988/87	46	24	21 (88)	9	1
1987/86	1	1	1 (100)	0	0
1986/85	396	139	136 (98)	50	19
1985/84	395	34	34 (100)	1	0
1984/83	49	25	24 (96)	7	2
1983/82	684	128	126 (98)	25	4
1982/81	399	90	83 (92)	19	2
1981/80	208	19	16 (84)	6	0
1980/79	317	46	46 (100)	20	0
1979/78	0	0	0	0	0
1978/77	692	10	5 (50)	1	0
1977/76	926	19	9 (47)	5	0
1976/75	1923	24	24 (100)	19	0
1975/74	941	4	3 (75)	1	0
1974/73	575	3	3 (100)	2	0
1973/72	1290	2	2 (100)	0	0
TOTAL	**13219**	**1771**	**1559 (88%)**	**278 (18%)**	**37 (2.4%)**

[1] Represents the actual number of influenza A H3N2 viruses isolated in the National Influenza Laboratory, Colindale over that particular influenza season. [2] Represents the number of viruses from that season tested by the drug susceptibility EIA in this study. [3] Represents the number of viruses that produced sufficient signal in the EIA to be analysed for drug susceptibility.

TABLE 3.5 Amantadine susceptibility screening data for influenza A H1N1 viruses

Season	No. isolated [1]	No. Tested [2]	No. Tested Virus Analysed (%) [3]	No. Resistant by EIA	No. Resistant by Plaque Assay
1999/98	0	0	0	0	0
1998/97	262	53	50 (94)	3	0
1997/96	8	3	3 (100)	0	0
1996/95	119	19	15 (79)	0	0
1995/94	8	4	4 (100)	0	0
1994/93	0	0	0	0	0
1993/92	73	34	22 (65)	2	0
1992/91	70	39	31 (79)	5	3
1991/90	3	1	1 (100)	0	0
1990/89	0	0	0	0	0
1989/88	257	97	75 (77)	9	1
1988/87	33	24	21 (88)	8	0
1987/86	315	102	97 (95)	24	1
1986/85	178	0	0	0	0
1985/84	16	10	9 (90)	0	0
1984/83	196	100	100 (100)	18	6
1983/82	182	81	80 (99)	8	0
1982/81	4	3	3 (100)	0	0
1981/80	177	10	3 (100)	3	0
1980/79	10	0	0	0	0
1979/78	72	11	5 (45)	2	0
1978/77	297	31	12 (39)	5	0
TOTAL	**2280**	**622**	**531 (85%)**	**87 (17%)**	**11 (2.1%)**

[1] Represents the actual number of influenza A H1N1 viruses isolated in the National Influenza Laboratory, Colindale over that particular influenza season. [2] Represents the number of viruses from that season tested by the drug susceptibility EIA in this study. [3] Represents the number of viruses that produced sufficient signal in the EIA to be analysed for drug susceptibility.

TABLE 3.6 Amantadine susceptibility screening data for influenza A H2N2 viruses

Season	No. Isolated [1]	No. Tested [2]	No. Tested Virus Analysed (%) [3]	No. Resistant by EIA	No. Resistant by Plaque Assay
1967/68	ND	3	2(66)	0	0
1966/67	ND	1	0	0	0
1965/66	ND	1	1(100)	0	0
1964/65	ND	7	7(100)	0	0
1963/64	ND	6	6(100)	0	0
1962/63	ND	23	22(96)	0	0
1961/62	ND	3	2(66)	0	0
TOTAL	**ND**	**44**	**40(90.9%)**	**0**	**0**

[1] Represents the actual number of influenza A H2N2 viruses isolated in the National Influenza Laboratory, Colindale over that particular influenza season. [2] Represents the number of viruses from that season tested by the drug susceptibility EIA in this study. [3] Represents the number of viruses that produced sufficient signal in the EIA to be analysed for drug susceptibility. ND – no data available.

3.3.3 Phenotypic Characterisation of Amantadine-Resistant Strains

Susceptibility testing by plaque reduction assay was performed on isolates that had been determined as resistant from the EIA screening. Of the 365 viruses resistant by the EiA, 48 (13.2%) were confirmed using plaque reduction assay. Although labour intensive, it provided valuable data concerning the phenotypic properties of the viruses.

The number of plaques formed in the presence and absence of amantadine by each virus was counted and the plaque forming units per ml (PFU/ml) calculated (Equation 2.1; page 41). It was then possible to determine the reduction in PFU/ml caused by the presence of amantadine; this figure was used to confirm the susceptibility of the virus (Table 3.7).

TABLE 3.7 Phenotypic characterisation of amantadine-resistant strains

Virus	Subtype	Season	PFU/ml		% Reduction	Plaque Size (mm)		% Reduction	Range (mm)	
			- Drug	+ Drug*		- Drug (95% CI)**	+ Drug* (95% CI)		- Drug	+ Drug*
A/ENG/798/98	H3N2	98/99	$9x10^2$	$6x10^2$	33.3	3.7 (± 0.9)	2.2 (± 0.4)	40.5	2-5	1.5-3
A/ENG/692/98	H3N2	98/99	$3.5x10^2$	$2.5x10^2$	28.6	2.3 (± 1.0)	0.7 (± 0.6)	69.6	1.5-3	0.3-1
A/ENG/424/98	H3N2	97/98	$2.5x10^3$	$2x10^3$	20	1.7 (± 0.8)	0.7 (± 0.8)	58.8	1-2.5	1-2
A/ENG/357/96	H3N2	96/97	$2.3x10^7$	$2.1x10^7$	9	2.2 (ND)	3.1 (ND)	-41	1.5-3	1-3.5
A/ENG/280/93	H3N2	93/94	$9x10^6$	$5.5x10^6$	38.9	3.4 (± 0.3)	1.6 (± 0.4)	52.9	3-4	1-2.5
A/SCOT/87/92	H3N2	91/92	No Data Available							
A/SCOT/77/92	H1N1	91/92	$6.5x10^6$	$5.5x10^6$	15.4	1.6 (± 0.4)	1.4 (± 0.3)	12.5	1-2.5	1-2
A/SCOT/62/92	H1N1	91/92	$6x10^5$	$4.5x10^5$	25	2.2 (± 0.7)	1.2† (± 0.3)	45.5	1-4	1-1.5
A/SCOT/59/92	H3N2	91/92	$2.6x10^6$	$3.7x10^6$	-42.3	1.5 (ND)	1.5† (ND)	0		
A/ENG/39/92	H3N2	91/92	$8.5x10^6$	$1.1x10^7$	-29.4	0.5 (± 0)	1.6 (± 0.4)	-220	0.5	1-2
A/ENG/327/90	H1N1	89/90	No Data Available							
A/ENG/129/89	H3N2	88/89	$1.3x10^3$	$1.7x10^3$	-30.8	2.1 (± 0.6)	2.2 (± 0.7)	-4.8	1-3.5	1-4
A/ENG/128/89	H3N2	88/89	$6x10^6$	$6x10^6$	0	3.3 (± 0.7)	1.7 (± 0.4)	48.5	2-4.5	1-2
A/SCOT/18/88	H3N2	88/89	$2x10^4$	$3x10^4$	-50	3 (± 0.6)	2.7 (± 0.7)	10	2.5-3.5	1.5-3.5
A/ENG/14/87	H1N1	86/87	$1.2x10^7$	$6x10^6$	50	2.9 (± 0.5)	1.1 (± 0.3)	62.1	1.5-4	1-2

*** Amantadine incorporated into agar overlay at 1µg/ml (final concentration). ** 95% confidence intervals. † Opaque plaques (+ drug). ND – not done.**

TABLE 3.7 Phenotypic characterisation of amantadine-resistant strains (continued)

Virus	Subtype	Season	PFU/ml		% Reduction	Plaque Size (mm)		% Reduction	Range (mm)	
			- Drug	+ Drug*		- Drug (95% CI)**	+ Drug* (95% CI)		- Drug	+ Drug*
A/ENG/638/86	H3N2	85/86	$1.1x10^6$	$7.7x10^5$	30	1.4 (ND)	0.6 (ND)	57.1	0.5-1.5	0.5-1
A/ENG/637/86	H3N2	85/86	$2.5x10^4$	$2x10^4$	20	1 (± 0.1)	0.6 (± 0.4)	40	0.5-2	0.5-1
A/ENG/632/86	H3N2	85/86	$3.6x10^5$	$3.3x10^5$	8.3	0.9 (± 0.1)	1.1 (± 0.1)	-22.2	0.5-1.5	0.5-1.5
A/ENG/629/86	H3N2	85/86	$5x10^5$	$4.5x10^5$	10	0.9 (± 0.5)	1.7 (± 0.7)	-88.9	0.5-2.5	1-2.5
A/ENG/589/86	H3N2	85/86	$1.5x10^6$	$1x10^6$	33.3	0.8 (ND)	1 (ND)	-25	0.5-1	1
A/ENG/588/86	H3N2	85/86	$5.8x10^6$	$5.8x10^6$	0	1.1 (± 0.2)	0.8 (± 0.2)	27.3	0.5-1.5	0.5-1
A/ENG/580/86	H3N2	85/86	$1.9x10^5$	$1.2x10^5$	36.8	0.7 (± .01)	0.9 (± 0.2)	-28.6	0.2-1.5	0.5-1.5
A/ENG/557/86	H3N2	85/86	$3x10^6$	$2x10^6$	33.3	0.5 (ND)	0.4 (ND)	20	0.2-1	0.2-0.5
A/ENG/550/86	H3N2	85/86	$6x10^5$	$6x10^5$	0	1 (± 0.3)	1.1 (± 0.2)	-10	0.5-1.5	0.5-1.5
A/ENG/549/86	H3N2	85/86	$2.1x10^5$	$1.4x10^5$	33.3	1 (± 0.1)	1.2 (± 0.1)	-20	0.5-1.5	1-1.5
A/SCOT/49/86	H3N2	85/86	$5.8x10^5$	$4.3x10^5$	25.9	1.5 (± 0.2)	0.8 (± 0.1)	46.7	0.5-2	0.5-1
A/ENG/395/86	H3N2	85/86	$3.8x10^7$	$3x10^7$	21.1	0.8 (± 0.1)	0.8 (± 0.1)	0	0.5-1	0.5-1
A/ENG/400/86	H3N2	85/86	$4.5x10^6$	$3x10^6$	33.3	1 (± 0.4)	0.8 (± 0.3)	20	0.5-2	0.5-1
A/ENG/310/86	H3N2	85/86	$1.6x10^5$	$1.2x10^5$	25	2.1 (± 0.3)	0.9 (± 0.2)	57.1	1-2.5	0.5-1.5
A/ENG/277/86	H3N2	85/86	$8x10^7$	$3.4x10^7$	57.5	0.9 (± 0.1)	0.7 (± 0.1)	22.2	0.5-1.5	0.5-1
A/ENG/268/86	H3N2	85/86	$5.3x10^5$	$4.3x10^5$	18.9	0.6 (± 0.1)	0.6 (± 0.1)	0	0.5-1	0.5-1
A/ENG/207/86	H3N2	85/86	$2.2x10^7$	$1.2x10^7$	45.5	1.4 (± 0.2)	0.7 (± 0.1)	50	1-3	0.5-1

*** Amantadine incorporated into agar overlay at 1µg/ml (final concentration). ** 95% confidence intervals. ND – not done.**

TABLE 3.7 Phenotypic characterisation of amantadine-resistant strains (continued)

Virus	Subtype	Season	PFU/ml		% Reduction	Plaque Size (mm)		% Reduction	Range (mm)	
			- Drug	+ Drug*		- Drug (95% CI)**	+ Drug* (95% CI)		- Drug	+ Drug*
A/ENG/168/86	H3N2	85/86	$4.7x10^7$	$3.1x10^7$	34	1.8 (± 0.2)	0.8 (± 0.1)	55.6	0.5-2	0.5-1
A/ENG/50/86	H3N2	85/86	$9x10^6$	$8x10^6$	11.1	1.2 (± 0.1)	1.1 (± 0.2)	8.3	0.5-1	0.5-1.5
A/ENG/221/84	H1N1	83/84	$9.3x10^3$	$7.3x10^3$	21.5	1.2 (± 0.1)	1.2 (± 0.1)	0	1-1.5	1-1.5
A/ENG/136/84	H1N1	83/84	$6.1x10^5$	$3.6x10^5$	41	1.4 (± 0.1)	1.4 (± 0)	0	0.5-1.5	1-1.5
A/ENG/134/86	H1N1	83/84	$4.4x10^7$	$3.4x10^7$	22.7	1.5 (± 0.2)	1.2 (± 0.1)	20	0.5-2	0.5-1.5
A/ENG/147/84	H1N1	83/84	$2.8x10^5$	$1.8x10^5$	35.7	0.8 (± 0.1)	0.8 (± 0.1)	0	0.5-1	0.5-1
A/ENG/118/84	H1N1	83/84	$6.7x10^5$	$5.7x10^5$	14.9	1.5 (± 0)	0.9 (± 0.1)	40	1.5	0.5-1
A/ENG/116/84	H3N2	83/84	$5.5x10^6$	$4x10^6$	27.3	1 (ND)	1.8 (ND)	-80	0.5-1.5	1.5-2
A/SCOT/76/84	H3N2	83/84	$2x10^5$	$1.7x10^5$	15	3.6 (± 0.2)	2.1 (± 0.2)	41.7	2.5-4	1.5-4
A/ENG/907/83	H3N2	83/84	No Data Available							
A/ENG/640/82	H3N2	81/82	$5.5x10^6$	$7.5x10^6$	-36.4	1.8 (± 0.4)	1.5 (± 0.3)	16.7	0.5-2.5	1-2.5
A/ENG/627/83	H3N2	82/83	$4x10^6$	$3x10^6$	25	1.4 (± 1.4)	1.3 (± 1.3)	7.1	0.5-2	1-1.5
A/ENG/259/83	H3N2	82/83	$3.1x10^5$	$2.9x10^5$	6.5	1.9 (± 0)	1.1 (± 0.1)	42.1	1.5-2	0.5-1.5
A/ENG/1/83	H3N2	82/83	$1.5x10^7$	$8x10^6$	46.7	0.6 (± 0.1)	0.6 (± 0.1)	0	0.5-1	0.5-1
A/SCOT/16/83	H3N2	82/83	$1x10^6$	$3x10^6$	-200	2.5 (± 0)	0.9 (± 0.2)	64	2.5	0.5-1
A/ENG/551/82	H3N2	81/82	$2.4x10^7$	$2.1x10^7$	12.5	3.3 (± 0.2)	1.3 (± 0.1)	60.6	1-3.5	1-1.5

*** Amantadine incorporated into agar overlay at 1µg/ml (final concentration). ** 95% confidence intervals. ND – not done.**

Initial analysis of the results indicated that there were wide ranges in infectivity titres of the viruses tested, from 3.5 x 10^2 PFU/ml (A/ENG/692/98) up to 8 x 10^7 PFU/ml (A/ENG/277/86). This observation was predicted because the viruses had been isolated from original archived material that contained different titres of virus. Of greater importance was the antiviral effect of amantadine on the PFU/ml of each virus. When the percent reduction in PFU/ml was calculated there was a range in the degree of resistance shown by the viruses. Resistance could be defined into two categories, high resistance and partial resistance. There were 15/48 viruses that appeared to be partially resistant (26-50% reduction PFU/ml) to amantadine and 21/48 viruses with high resistance (1-25% reduction PFU/ml). The remaining viruses (6/48) had negative values for percent reduction in PFU/ml indicating that there had been an increase in the PFU/ml in the presence of drug. This result had not been expected; the increase of viral growth in the presence of amantadine had not previously been reported.

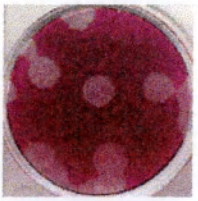
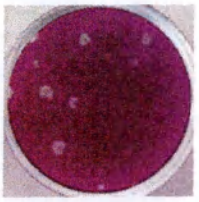
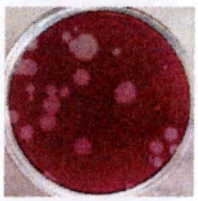
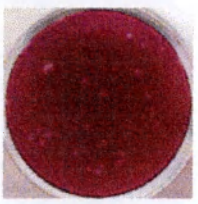
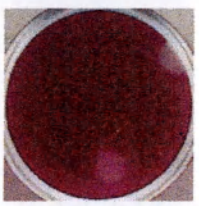
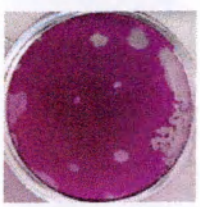
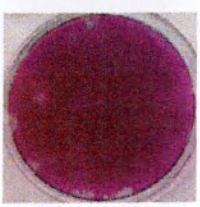
The phenotypic characteristics of each virus were analysed and recorded. The diameter of each plaque formed was measured and any interesting morphological features noted. The mean plaque size in the presence and absence of amantadine and the percent reduction in size of plaque in the presence of drug were calculated for each virus (Table 3.7). From these results it was possible to estimate the growth potential and look for the presence of heterogeneous populations of infective virus particles within the isolate. Plaques were noted for 45/48 viruses (94%). The remaining viruses could not be analysed because of poorly defined plaques however it was possible to identify endpoints of viral growth and therefore estimate the susceptibility of the virus to amantadine. Results indicated that for the majority of viruses (80%), plaque size was reduced in the presence of amantadine. The reduction in diameter of plaques ranged from 7.1% to 69.6%. This suggested that resistant viruses growing in the presence of amantadine had a reduced growth potential when compared to the sensitive population of virus, causing the formation of smaller plaques. A small group of viruses (7/48) had plaques that were identical in size in the presence and absence of amantadine. This implied that although amantadine had reduced the PFU/ml, the growth characteristics of the resistant viruses were identical to the sensitive. In order to further investigate this observation, it is proposed that plaques grown in the presence of amantadine could be picked and used in one-step growth curve experiments to determine the rate of replication and virus yield. This would ascertain whether the sensitive and

resistant viruses with similar plaque morphologies were indeed similar in replication and virus yield.

An interesting observation was made where several viruses had formed larger plaques in the presence of amantadine (Table 3.7). In total 10/48 (21%) viruses demonstrated this phenomenon but some showed a more substantial increase than others. The viruses A/ENG/116/84, A/ENG/629/86 and A/ENG/39/92 had an 80%, 88.9% and 220% increase in plaque size respectively. This was interesting because it suggested that in the presence of drug, the resistant population of virus that had been selected had much greater growth potential than the sensitive population in presence of drug.

Another interesting morphological feature observed was the formation of opaque plaques; two viruses (A/SCOT/62/92 and A/SCOT/59/92) displayed this phenomenon. In the absence of drug the plaques appeared normal with complete removal of the cell sheet but growing virus in the presence of drug appeared to form a mixed population of clear and opaque plaques. Closer inspection of the individual plaques revealed that although the majority of the cell monolayer within the boundary of the plaque had been infected, the virus had not caused the cells to be completely removed leaving a layer of cells that gave the plaque its opaque appearance. The plaques formed in the presence of drug were identical in size for A/SCOT/59/92 and 45.5% smaller for A/SCOT/62/92 to plaques formed in the absence of drug. A selection of resistant viruses from the current study displaying the above phenotypic and morphological characteristics is presented in Table 3.8.

TABLE 3.8 Observed phenotypic characteristics of selected resistant viruses

Virus	Observed Characteristic	Plaque Morphology	
		- Drug	+ Drug*
A/ENG/14/87	Reduction in plaque size		
A/ENG/207/86	Selection of plaques from heterogeneous population		
A/ENG/395/86	Small plaque size -/+ drug		
A/SCOT/18/88	Large plaque size -/+ drug		
A/ENG/357/96	Increase in plaque number + drug		
A/ENG/39/92	Increase in plaque size + drug		
A/SCOT/62/92	Opaque plaques + drug		 †

*** Amantadine added to agar overlay at 1.0µg/ml (final concentration). † Opaque plaques are difficult to visualise.**

3.3.4 Epidemiological and Clinical Analysis of Amantadine-Resistant Strains

Initial analysis of data from laboratory reports revealed that the origins of the resistant isolates were spread over the UK. The majority of isolates had been spread over England with several originating from areas around Scotland (Table 3.9). There was evidence to show that some individual areas were associated with more resistant viruses than others, e.g. Guildford and Bath. It was hypothesised that these clusters of resistant viruses might have originated from small outbreaks. Further analysis of the data revealed that there were some viruses that could be grouped, and classed as outbreak cases. The most significant example was a group of 1985/86 viruses that originated from Guildford Public Health Laboratory. Examination of laboratory details determined that the patients from which samples had been taken were all adolescent males suggesting that they may have originated from a school or institution. Dates of sampling from this specific case showed that eight isolates had all been received within approximately 10 weeks. Three of the samples had been collected within a period of 14 days and four had been received on two dates, with two viruses isolated on each day. Further investigation revealed that this case was a boarding school outbreak.

Another interesting case involved four viruses that were isolated from Scotland (Table 3.9). Initially, it was hypothesised that these strains would all have originated from the same location at approximately the same time suggesting an outbreak case involving the transmission of a resistant strain. However, analysis of laboratory details revealed that all of the isolates had been separately isolated from different regions of Scotland at different times indicating that these were unrelated, sporadic cases.

TABLE 3.9 Epidemiological analysis of amantadine-resistant strains

Virus	Season	Subtype	Origin	Date of Isolate	Original Specimen
A/ENG/798/98	98/99	H3N2	Maidenhead	29/12/98	TS
A/ENG/692/98	98/99	H3N2	Plymouth	22/12/98	TS
A/ENG/424/98	97/98	H3N2	London	19/03/98	TCF
A/ENG/357/96	96/97	H3N2	Bristol	17/12/96	TCF
A/ENG/280/93	93/94	H3N2	Preston	27/10/93	TS
A/SCOT/87/92	91/92	H3N2	Glasgow	01/05/92	TCF
A/SCOT/77/92	91/92	H1N1	Aberdeen	05/03/92	TCF
A/SCOT/62/92	91/92	H1N1	Glasgow	18/02/92	TCF
A/SCOT/59/92	91/92	H1N1	Edinburgh	14/02/92	TCF
A/ENG/39/92	91/92	H3N2	Leeds	13/01/92	TCF
A/ENG/327/90	89/90	H3N2	Leicester	20/02/90	TCF
A/ENG/129/89	88/89	H3N2	Preston	31/01/89	TCF
A/ENG/128/89	88/89	H3N2	Manchester	31/01/89	TCF
A/SCOT/18/88	88/89	H3N2	Edinburgh	05/05/88	TCF
A/ENG/14/87	86/87	H1N1	Chelmsford	23/01/87	TCF
A/ENG/638/86*	85/86	H3N2	Nottingham	16/05/86	TCF
A/ENG/637/86*	85/86	H3N2	Nottingham	16/05/86	TCF
A/ENG/632/86*	85/86	H3N2	Guildford	09/05/86	TCF
A/ENG/629/86*	85/86	H3N2	Guildford	09/05/86	TCF
A/ENG/589/86	85/86	H3N2	Newcastle	22/04/86	TCF
A/ENG/588/86	85/86	H3N2	Stoke	22/04/86	TCF
A/ENG/586/86	85/86	H3N2	Newcastle	17/04/86	TCF
A/ENG/557/86	85/86	H3N2	Manchester	09/04/86	TCF
A/ENG/550/86*	85/86	H3N2	Guildford	09/04/86	TCF
A/ENG/549/86*	85/86	H3N2	Guildford	09/04/86	TCF
A/SCOT/49/86	85/86	H3N2	Edinburgh	26/03/86	TCF
A/ENG/395/86*	85/86	H3N2	Guildford	13/03/86	TCF
A/ENG/400/86*	85/86	H3N2	Guildford	14/03/86	TCF
A/ENG/277/86*	85/86	H3N2	Guildford	03/03/86	TCF
A/ENG/310/86	85/86	H3N2	Bath	04/03/86	TCF
A/ENG/268/86	85/86	H3N2	Preston	03/03/86	TCF
A/ENG/207/86	85/86	H3N2	Guildford	26/02/86	TCF
A/ENG/168/86	85/86	H3N2	Nottingham	19/02/86	TCF
A/ENG/50/86	85/86	H3N2	Bath	24/01/86	TCF
A/ENG/221/84	83/84	H1N1	Newcastle	16/04/84	TCF
A/ENG/136/84*	83/84	H1N1	Bath	02/04/84	TCF
A/ENG/134/84*	83/84	H1N1	Bath	02/04/84	TCF
A/ENG/116/84	83/84	H3N2	Birmingham	30/03/84	TCF
A/ENG/147/84	83/84	H1N1	Guildford	02/04/84	TCF
A/ENG/118/84	83/84	H1N1	Coventry	30/03/84	TCF
A/SCOT/76/84	83/84	H1N1	Glasgow	05/06/84	TCF
A/ENG/907/83	83/84	H3N2	Manchester	18/10/83	TCF
A/ENG/627/83	82/83	H3N2	Bath	17/03/83	TCF
A/SCOT/16/83	82/83	H3N2	Edinburgh	15/02/83	TCF
A/ENG/259/83	82/83	H3N2	Nottingham	14/02/83	TCF
A/ENG/1/83	82/83	H3N2	Newcastle	05/01/83	TCF
A/ENG/640/82	81/82	H3N2	Sheffield	07/04/82	TCF
A/ENG/551/82	81/82	H3N2	Leicester	01/04/82	TCF

TCF - tissue culture fluid; TS - throat Swab; * - possible outbreak cases.

3.4 Discussion

This study documented amantadine-resistance in human influenza A viruses isolated over the last century. The viruses circulated within a geographically-contained location with defined seasonal influenza. Previous studies have been concerned with the isolation of viruses during and after treatment (25, 71, 147, 166, 193, 238) but few have been able to ascertain the frequency of resistance in non-treated individuals (156, 277, 417). Here, an estimate of amantadine-resistance in a geographically discreet population was made. The use of amantadine within the UK had been limited, therefore this provided a more accurate representation of natural emergence and variation of amantadine susceptibility in influenza A viruses.

3.4.1 Methodology Conclusions

All various methods described to assay antiviral drugs are based on measuring the viral growth, and inhibition of viral growth in the presence of the inhibitor, but this can be achieved in several ways. The plaque reduction assay measures the production of progeny infectious virus particles detected by the formation of plaques. The EIA is designed to detect the presence of virus-specific antigens produced during the replication cycle of the virus. The use of EIA in determining the susceptibility of viruses to antiviral compounds is acceptable as long as there is a direct relationship between the production of virus-antigen and infectious virus. For example, an antiviral compound targeting a late stage of the virus replication cycle will not affect the synthesis of antigens produced early in the cycle. Therefore, when designing an antiviral susceptibility testing strategy, it is important to understand the different interactions of virus and drug.

The methodology used in this study involved the design and optimisation of an EIA to determine susceptibility of influenza isolates to amantadine. The ability of the assay to perform this task was validated by the results gained however it was apparent from the discrepancy between the EIA and plaque reduction assay results that the EIA was selecting a large number of 'false-resistant' viruses. The percentage of H3N2 and H1N1 viruses selected by the EIA as resistant that were confirmed resistant by plaque reduction assay was 13.3% and 12.6% respectively.

The apparent low fidelity of the amantadine-susceptibility screening EIA suggests that further work is required to fully optimise the assay. The aim of this section of the work was to design and utilise an assay that would enable a large number of viruses to be screened for drug susceptibility. The antiviral testing 'gold-standard' (plaque reduction assay) was then used to confirm the drug phenotype of EIA-resistant viruses. It was possible that in an attempt to fully maximise the efficiency of the EIA, i.e. increase the number of viruses per assay and reduce the test turnaround time, the specificity of the assay had been compromised, the overall specificity of the assay was estimated to be approximately 87%. In order for the assay to be efficiently used within a routine diagnostic laboratory, where high throughput of samples for drug-resistance testing would possibly not be a priority, further optimisation is required to reduce the rate of false-resistant results. Therefore, it might be concluded that in this respect the assay was successful, it tested over 2300 clinical samples and selected amantadine-resistant strains, but in order to improve the overall specificity more time would have to be invested to 'fine tune' certain components of the assay.

The reasons why the specificity of the assay was relatively low were not clear but it was possible that the assay had become over-sensitive at detecting resistant viruses. To perform the test, virus samples were serially diluted from a ten-fold to 1000-fold dilution. It was concluded that this dilution was not sufficient for viruses with extremely high titres. It appeared that the viruses were growing equally in both media supplemented with and absent of drug. It was possible that the EIA was over-sensitive and where virus had been inoculated onto cells in the presence of drug at a high multiplicity of infection, there was a high proportion of non-infectious viral debris that remained attached to and within the cells after washing stages. This material would have provided a substrate for antibody to bind to throughout stages of the assay resulting in a falsely high optical density reading that implied that the virus had undergone infectious cycles in the presence of the drug. To overcome this limitation, viruses should have been diluted further to enable a lower multiplicity of infection to be achieved. In this situation there would have been sufficient quantity of virus to infect the cell monolayer without having an excess of non-infectious virus remaining in association with the cells throughout the test. Unfortunately, to modify the assay in this way would have reduced its capability of having a high throughput of viruses.

The methodology involved with the drug-susceptibility screening EIA used in this study was compared to other EIA methods from similar studies (23, 25, 147). Overall, the different methodologies used were comparable, however, there were some small discrepancies. For example, in the present study an anti-influenza A nucleoprotein antibody was used to detect viral antigen, other studies used polyclonal ferret antiserum raised against either H3N2 or H1N1 virus. It is unclear how this might affect the overall specificity of the assay, but it is a viable option for future work to optimise the assay. Interestingly, one of the studies using a similar drug-susceptibility EIA diluted viruses 10^{-1} to 10^{-6} in ten-fold serial dilutions (23). As discussed above, it was possible that the apparent low specificity of the EIA from the present study was a result of viruses at high titres that had not been sufficiently diluted when used in the assay. Therefore, the methods used by Belshe *et al.* (1989) confirm that if the assay is to be further optimised, dilution of viruses to 10^{-6} might improve the specificity of the test.

3.4.2 Frequency of Resistance

The frequency of amantadine-resistance of influenza A viruses screened in this study was 2.4%, 2.1% and 0% for H3N2, H1N1 and H2N2 subtypes, respectively. This resulted in an overall frequency of 2.25% for influenza A viruses. Due to the nature of the study and subjects, this figure was considered to represent an approximation of the level of resistance occurring in the UK in non-treated individuals. There was evidence to suggest that some of the isolates tested were from small outbreaks arising as a result of amantadine use. In order to calculate the natural frequency as accurately as possible, the data for these suspected viruses were omitted and the figure recalculated; this adjusted figure was calculated to be 1.7% for all subtypes. When compared to the most recent susceptibility study published, where the frequency of amantadine-resistance was determined to be 0.8%, the figure generated from the present study both including and excluding suspected outbreaks was higher (417). The majority of resistant viruses (14/16) isolated by Zeigler *et al.* (1999) could not be associated with any form of drug treatment and it was concluded that these viruses had emerged as a result of the high mutation rate of the virus, generating the changes within M2 necessary to confer resistance. However, the study investigated the occurrence of resistance in 43 countries globally and therefore did not give a true picture of

susceptibility in one population. When the results from the study were broken down into the different countries there were some interesting observations. The majority of isolates tested originated from the US (n=991); the frequency of resistance was calculated at 0.8%. There was no resistance detected in viruses isolated in China (n=150), but the most interesting finding was isolates that had originated from Australia. In total, 94 isolates were tested and 5 (5.3%) were detected as resistant. All five resistant isolates were found to be from unrelated individual cases, similar to the majority of the viruses isolated in the present study. Further data by Zeigler *et al.* (1999) revealed that over a total of 6 years in Australia, 9/198 (4.5%) isolates were found to be drug-resistant (417). This was a relatively high figure for resistance but more significantly Australia was very similar to the UK in respect of the extremely limited use of amantadine within the population for treating influenza. Within this relatively small, contained population there was evidence that resistant viruses had circulated without significant selective pressure from amantadine or rimantadine. This finding correlated with the results presented here for the UK where unrelated individual cases of resistant viruses were isolated. Therefore, using data from this present study and previous investigations (156, 417) it was concluded that the occurrence of resistant viruses in an environment where the use of amantadine or rimantadine for the treatment of influenza infections was limited were predominantly as a result of the high mutation rate of the influenza virus, but with a small proportion resulting from individual isolated cases of amantadine use. This was true of the UK where amantadine has been limited to treatment within residential homes and schools and is rarely used even in these situations. Therefore selective pressure from the drug on the virus was extremely low meaning that there must have been some other mechanism i.e. spontaneous mutation, to force the emergence of the resistant viruses.

Results from the screening of viruses by the susceptibility EIA demonstrated that within the H3N2 and H1N1 subtypes there was variation in the susceptibility of viruses to amantadine (data not shown). Viruses that were drug-sensitive (<50% reduction growth) showed a range in susceptibility in the presence of drug as demonstrated by a variety of differing OD readings. These varied from highly susceptible, to viruses of intermediate susceptibility that were only slightly above the cut-off for resistance. This demonstrated that the H3N2 and H1N1 influenza

viruses tested varied in some way that determined their susceptibility to amantadine.

These results correlated with findings from a previous study conducted by Pemberton *et al.* (1986) who tested the susceptibility of H1N1 and H3N2 viruses against amantadine (277). They found viruses had a range of susceptibilities when titrated against amantadine. The biological cloning of a highly resistant virus produced a set of clones that were found to have a range of susceptibilities to drug. It was suggested that this was as the result of the presence of heterogeneous populations within any one virus isolate. It has previously been demonstrated that any single influenza virus preparation can contain a complex heterogeneous population of variants (quasispecies) that will include viruses of both resistant and sensitive genotype (193). It was therefore proposed that sensitive strains of influenza A could contain differing proportions of resistant genotypes within the quasispecies. A strain containing a relatively high proportion of resistant genotypes would have a lower susceptibility to amantadine compared to a strain containing a population of predominantly sensitive genotypes. This quasispecies theory may provide an explanation for the difference in susceptibility of viruses observed in this study. The H3N2 and H1N1 subtypes have been in circulation during a period when amantadine has been in use worldwide, but in limited use within the UK. This limited exposure to the drug may have had some influencing effect on the levels of susceptibility experienced in this study. Any selective pressure from amantadine might have had an effect on the balance of genotypes within the viral quasispecies. This might not have been initially obvious because the overall phenotype of the virus would remain the same, i.e. sensitive, but the actual susceptibility of the virus would be reduced.

Evidence to support this hypothesis was evident within some of the phenotypic data collected by plaque reduction assay. It was noted that several virus preparations produced different sizes of plaques when grown without drug e.g. A/ENG/207/86 but when the virus was grown in the presence of amantadine small plaques were selected (Tables 3.7 and 3.8). This suggested that without the selective pressure of drug, a viral quasispecies existed that consisted both of sensitive and resistant genotypes, where the sensitive genotypes maintained a selective advantage over the resistant genotype. However, when a selective pressure was applied to the quasispecies in the form of amantadine, it suppressed

replication of the sensitive genotypes and allowed the resistant genotype to grow resulting in the selection of a single plaque size. These results suggested that some virus preparations were composed of mixed heterogeneous populations containing differing genotypes that contributed to the variation in susceptibility observed.

In comparison, H2N2 viruses showed less variation with the actual level of susceptibility being significantly higher. This might be explained by the absence of amantadine use in the late 1960s when H2N2 viruses were circulating. There was no resistance detected in the H2N2 group indicating that there was very little selective pressure, if any, on the viruses causing them to specifically mutate within the M2 proteins and accumulate resistant genotypes within the quasispecies. However, one must be careful in drawing any conclusions from this set of viruses because only a small number of isolates were tested in proportion to the total number isolated. If the frequency of resistance determined for H3N2 and H1N1 viruses (2.3%) was the same for H2N2 viruses then in theory a minimum of approximately 50 viruses would need to have been screened before a resistant virus was discovered. It was also very difficult to generate conclusions because H3N2 viruses superseded H2N2 viruses in 1968/69; if H2N2 viruses had continued circulating within the population it would have been interesting to see whether their susceptibility to amantadine would have decreased as they gained more exposure to the drug. The hypothesis that H2N2 viruses were more susceptible to amantadine because they were less exposed to the drug is not supported by other data (175). Some influenza viruses e.g. A/WSN/33 and A/PR/8/34, with parental strains isolated before the use of amantadine have been shown to be drug-resistant (175). The exact mechanism by which these viruses acquired resistance is not clear but it provides evidence that resistant viruses can be generated without the selective pressure of drug.

3.4.3 Variation of Amantadine Susceptibility Between Influenza Seasons

The screening results for H3N2 and H1N1 viruses demonstrated that there were different levels of resistance between influenza seasons. This was evidence to support the hypothesis that variation in susceptibility had occurred. An interesting pattern observed in the screening results was the occurrence of clusters of

resistance within certain seasons. These clusters suggested that there was some natural variation within the susceptibility of viruses circulating in the population each influenza season. An influencing factor to be considered was the proportional sampling of viruses each season; the number of viruses screened was dependent on the available viruses in the archive. From Tables 3.4 and 3.5 it can be seen that the number of viruses tested for each season varies greatly. This might have significantly influenced the results; the greater the number of isolates tested each season the more probable the isolation of a greater number of resistant viruses. The presence of amantadine-resistant strains was first detected (by plaque reduction assay) in the 1981/82 and 1983/84 influenza seasons for H3N2 and H1N1 subtypes, respectively. It was possible that this was a consequence of the relatively small number of viruses tested in the seasons leading up to the first isolation of drug-resistant viruses. Therefore, it was possible that the apparent lack of resistance preceding these dates was simply because the probability of isolating resistant viruses was lower. Unfortunately, all viruses available for testing over this period were analysed so there was no method of avoiding this sampling bias.

From data previously published it was hypothesised that viruses put in chronological order would display a gradual increase in levels of resistance. This evidence for this hypothesis originated from a study by Pemberton *et al.* (1986) who found an apparent increase in the level of resistance in viruses tested from 3 consecutive years (277). The proposed explanation for this finding was that the viruses had evolved in an environment where amantadine was present and therefore had become less susceptible over time, although it was conceded that the study had tested a significantly small collection of viruses compared to the number circulating the community. However, findings from the study presented here do not support this hypothesis. Levels of resistance, season to season, appeared to be irregular with spontaneous bursts or peaks at random times. The period that could be associated with a higher incidence of resistance was the 1980s where the most resistant viruses were isolated. The reasons behind this finding however were unclear.

3.4.4 Variation of Amantadine Susceptibility between Influenza Subtypes

There appeared to be no significant variation in resistance between H3N2 and H1N1 subtypes from amantadine-susceptibility screening; resistance was determined to be 2.4% and 2.1% respectively. There was however variation in susceptibility when these two subtypes were compared with the H2N2 viruses.

The contribution of virus subtype to the frequency of resistance to amantadine or rimantadine occurring in influenza A viruses has not been previously ascertained. A study by Kubar *et al.* (1989) investigated the efficacy of rimantadine in the treatment of outbreaks of influenza in the former Soviet Union (196). The aim of the work was to determine whether the development of resistant viruses had diminished efficacy of the drug. Over a period of seven years the study isolated rimantadine-resistant H1N1 (4.7%) and H3N2 (40.1%) variants (196). This finding illustrated that there were differences in the isolation of resistant viruses within influenza A subtypes.

Using results from the study presented here, it was possible to hypothesise the processes that might lead to variation in amantadine susceptibility between different antigenic subtypes of influenza A. The length of time that a virus circulated within a population would theoretically increase the number of replication rounds. This would increase the probability that mutations conferring amantadine-resistance would occur and therefore that the susceptibility to the drug would decrease. This might have explained the results from Kubar *et al.* (1989) where the H3N2 viruses tested had a high level of resistance (196). If the circulating strains had been predominantly H3N2 in the former Soviet Union then this would have allowed a greater duration of replication for the H3N2 viruses to accumulate mutations.

The present study indicates that the frequency of resistance was slightly higher for H3N2 viruses when compared to H1N1 viruses. Although this difference was small, the predominant circulation of H3N2 viruses within the UK may explain the finding. After the reappearance of the H1N1 subtype in 1977/78 there has been cocirculation of H3N2 and H1N1 viruses (Figure 3.2). However, the cocirculation has not been balanced; in the majority of seasons there was a predominance of one subtype, sometimes completely lacking any activity of the other. In general,

the number of isolations of H3N2 was greater within the UK indicating that the duration of circulation has been more prolonged. In effect this has increased the length of time the virus has been in circulation and therefore the time for the virus to evolve in respect to amantadine susceptibility.

The seasons where the greatest number of resistant viruses had been detected were characterised by a dominant subtype circulating and limited activity from the other. This trend was true except for the 1985/86 season where the distribution of circulation was approximately 69% and 31% for H3N2 and H1N1, respectively. All other seasons e.g. 1981/82 and 1983/84 where resistant viruses had been isolated had a predominance of either H3N2 or H1N1 circulating. Therefore it appeared that one influencing factor in the selection of resistant viruses of certain subtypes was the actual balance of subtypes within the season in question.

3.4.5 Phenotypic Analysis of Resistant Viruses

Data collected from plaque reduction assays were valuable in characterising the resistant viruses selected. Previous data collected from the EIA screening had suggested that there was variation in the level of susceptibility shown by sensitive viruses to amantadine. From infectivity data it appeared that resistant viruses had high and partial levels of resistance to amantadine. The presence of highly resistant strains suggested that these viruses contained heterogeneous populations that were predominantly comprised of resistant genotypes. It was speculated that the partially resistant strains would contain lower proportions of resistant genotypes mixed with sensitive particles that would result in a greater reduction in PFU/ml in the presence of amantadine. The plaque reduction assay results supported the theory that resistant viruses were also heterogeneous in their susceptibility to the drug. Overall, there was variation in the susceptibility of sensitive viruses, and within amantadine-resistant viruses, levels of resistance also varied.

An extremely interesting group of viruses was identified where certain viruses displayed an increase in PFU/ml in the presence of drug. This observation could not be found in any other previously published studies, although the phenomenon of drug dependence has been observed in influenza A viruses treated with the

neuraminidase inhibitor drug zanamivir (30, 242). Within other viruses, including poliovirus and human rhinovirus, data have also been reported indicating that drug dependence can occur (107, 377).

Results presented for the size of plaques produced by resistant viruses in the presence and absence of drug gave similar results to that from the PFU/ml data. There was variation in plaque size as a consequence of the presence of drug; viruses could be grouped into categories of high and partial resistance depending on the degree of inhibition shown by treatment of amantadine. Similar to the infectivity data there was a group of viruses that appeared to have larger plaques in the presence of amantadine indicating that the viruses were growing at increased rates. It was speculated that the virus populations contained within the larger plaques formed in the presence of drug had increased fitness in respect to growth properties. This was an interesting observation because the majority of viruses tested had an overall reduction in plaque size indicating that the resistant viruses able to propagate in the presence of drug had reduced growth potentials compared to the sensitive genotypes.

The data from PFU/ml and plaque size were analysed to determine whether there were any correlations between the figures. This analysis revealed that several viruses had distinct properties when grown in the presence of drug (A/ENG357/96, A/SCOT/59/92, A/ENG/39/92, A/ENG/129/89, A/ENG/632/86, A/ENG/629/86 and A/ENG/550/86). They all had increased PFU/ml and plaque size indicating that they had a preference for growing in 'drug-rich' conditions (Table 3.7).

It may be that the M1 species within these viruses differed in their biochemical properties. When amantadine blocks the M2 ion channel, it reduces the flow of protons from the acidic endosome into the interior of the virion (137). This process inhibits the low-pH disassembly of the ribonucleoprotein/M1 (RNP/M1) complex and therefore prevents the uncoating and release of the RNP into the host cell cytoplasm. If the M1 species of a virus had an altered pH tolerance, then this may have facilitated the dissociation of the RNP/M1 complex at a less acidic pH that would compensate for the blockage of the ion channel by amantadine. As a result of this, when the virus was propagated in the absence of amantadine, the internal pH of the virion would be too acidic and therefore not at the optimum pH for the dissociation of the RNP/M1 complex. The overall result would appear as an

increase in the PFU/ml and possibly the plaque size between viruses grown in the absence and presence of drug. Although only hypothetical, this theory could be tested by analysing the dissociation of the RNP/M1 complex at different pH values. Results from this experiment would give an indication of the optimum pH at which dissociation occurred. It would be interesting to perform this work using resistant viruses displaying different extremes of resistance, i.e. high reduction in PFU/ml and plaque size (e.g. A/ENG/168/86) and increase in PFU/ml and plaque size in the presence of drug (e.g. A/ENG/39/92).

3.4.6 Epidemiology of Resistant Viruses

The available clinical data associated with resistant viruses was extremely limited which unfortunately restricted the possible analysis. However, details of location, age and sex of isolates and patients were available. It was not possible to ascertain whether patients had exposure to amantadine either through treatment or contacts but evidence was gathered to predict where such cases had occurred.

The initial analysis revealed that the distribution of resistant viruses within the UK appeared to be random, viruses originated from locations in both the North and South of the country. However, it was important to consider that there was a certain bias in these findings; the distribution of the viruses was restricted to areas containing either a public health or hospital laboratory. This was certainly the case for older isolates where the majority were sent from such establishments, it was only in approximately the last five years studied that viruses were more evenly distributed as a result of community-based surveillance schemes being implemented (6, 97). This random distribution of resistance indicated that the majority of resistant viruses were sporadic, unrelated cases which was corroborated by conclusions drawn from the data presented concerning the frequency of resistance. There were groups of resistant viruses that were clustered into certain seasons. When epidemiological data was linked to these clusters it provided further evidence for the unrelated and sporadic nature of the majority of cases. Within a single season containing a cluster of viruses, e.g. 1983/84, viruses appeared to have a random distribution illustrating that they were unrelated cases. This was further supported by the analysis of four isolates originating from Scotland. Epidemiological data demonstrated that the viruses all

originated from separate hospital laboratories in Scotland. Due to the fact that the viruses were isolated from patients in hospitals, the viruses could have emerged as a result of individual treatment cases of amantadine or were unrelated cases from non-treated individuals. Unfortunately, laboratory reports did not specify any amantadine treatment that patients had undergone, therefore the reasons why these four viruses emerged can only be speculated.

There were however several interesting groups of viruses. Within the 1985/86 influenza season, which contained the largest collection of resistant viruses, there was evidence to suggest that a group of viruses were related. In particular, 8/19 viruses collected from 1985/86 originated from Guildford Public Health Laboratory. They were all H3N2 viruses, isolated within a period of approximately 10 weeks between February and May 1986. Analysis of laboratory reports revealed that the patients from whom viruses had been isolated were all adolescent males aged 12-16 years. This was interesting because it suggested that the viruses originated from a school or similar establishment. Evidence to support this theory was found in several reports documenting influenza outbreaks in boarding schools. Davies *et al.* (1988) studied an outbreak at Christ's Hospital boarding school where amantadine had been used prophylactically to control an outbreak of influenza (64). The first case was reported on 29/01/86 and laboratory confirmation of influenza H3N2 was provided by Guildford PHL. The outbreak persisted for approximately 56 days until the last confirmed isolation on 23/03/86. This data correlated with the epidemiological results presented in the present study. Viruses sent to Guildford for laboratory confirmation would only have been reported as influenza positive. To type the virus isolates, tissue culture fluid was forwarded to the Influenza Laboratory, CPHL where the subtype and antigenic profile of virus were determined. Therefore, this was evidence that the group of Guildford strains originated from a single school outbreak. In total, approximately 86 influenza viruses were isolated at CPHL from samples sent from Guildford; 8 viruses (9.3%) were determined as amantadine-resistant by plaque reduction assay when screened during this study.

The study by Davies *et al.* (1988) had revealed that a programme of amantadine prophylaxis had been initiated approximately one week after the first confirmed case of influenza at the school (64). Amantadine was taken by 79.2% of pupils; prophylaxis lasted for 14 days. During this period of amantadine use, 36 pupils

were diagnosed as having clinical influenza; 23 (64%) had been taking amantadine. Therefore, a number of pupils were receiving amantadine prophyiactically for a two-week period succumbed to an influenza infection (64). The evidence suggested that the prophylaxis failures within the school had been caused by the emergence of resistant viruses as a consequence of exposure of the virus to amantadine. It is possible that this could have been as a result of the emergence of an index resistant virus, e.g. (A/ENG/207/86) that was then transmitted to other pupils, or the emergence of several unique resistant viruses that then spread through the school population. The study by Davies *et al.* (1988), and the findings from the present study illustrate the difficulty of using amantadine in situations where there is close contact between individuals (64). The occurrence and transmission of resistance within similar environments has been documented and demonstrates the limitations of the drug (130, 147, 166). Matrix gene sequence analysis of the resistant strains associated with known and suspected outbreak cases will provide evidence for the transmission of the viruses. If all strains appear to be homogeneous in amino acid sequence then this would suggest that transmission of resistant viruses had occurred.

There was also some evidence that several of the viruses that had been received from Bath PHL could have originated from a boarding school. An earlier report had described the policy of treating pupils at the school with amantadine in preference to the trivalent vaccine; results during the study had demonstrated that superior protection was provided by amantadine (306, 307). This study showed that a proportion of pupils who had received amantadine had developed symptoms of influenza, which had subsequently been confirmed by laboratory analysis. This suggested that resistant viruses originating from Bath may have been as a direct consequence of amantadine treatment. It was unfortunate however, as the clinical details relating to the isolates received from Bath were extremely limited and the ages of the patients could not be found. This, with the available evidence above demonstrated that although the use of amantadine in the UK has been extremely limited, there have been isolated cases where it has been used to control outbreaks and small epidemics.

3.4.7 Transmission of Resistant Viruses

It has been documented that amantadine-resistant viruses can be transmitted person to person (147, 238) and it was hypothesised that the set of viruses originating from Guildford during the 1985/86 season supported this theory. The remaining results from this study however suggested that the frequency of transmission of resistant viruses in the general population was low. Evidence for this was apparent when seasonal data were examined. If the transmission of resistant viruses was a common event, then it would be expected that resistant viruses would spread quickly through a localised population similar to the pattern of wild-type sensitive viruses. The results from the screening suggested that resistant viruses emerged and then disappeared from the population. A good example could be seen where four viruses were isolated in Scotland from individual unrelated cases. Each virus had originated from a hospital laboratory and therefore it was concluded that the isolates would have originated from hospital wards. If there had been general transmission of the resistant viruses then it would have been expected that more resistant viruses would have been isolated. The confines of a hospital ward would have provided the ideal conditions for viral transmission between patients and therefore, the apparent lack of evidence for these events would indicate that transmission did not occur frequently.

In general, the pattern of emergence of resistance from this study did not support transmission of resistant viruses within a population. If resistant viruses were generally spread though a community or population then there should have been more evidence for this, especially during the more recent seasons screened. These periods contained a much higher proportion of viruses that originated from community-based surveillance schemes. If there was transmission of resistant viruses then these schemes should have identified more isolates of such viruses. This was not the case though; in the later years of the study, which included more community-based schemes, the frequency of resistance was lower than previous seasons. Therefore, the evidence would indicate that the population-based transmission of resistant viruses was extremely low. The transmission of resistant viruses has previously been documented, but only in situations where amantadine or rimantadine have been used in treatment or therapy (147, 238). These cases have also originated in either family or residential homes where there has been close contact between index cases and contacts. Therefore, in comparing results

from this study and previous investigations it would appear that the ideal conditions for transmission would be an environment where drug was in use, exerting a selective pressure on the virus, and where there is extremely close contact between patients. The transmission of resistant viruses amongst a population, it would appear, occurs at a low frequency.

3.4.8 Concluding Remarks

The overall level of amantadine-resistance in the UK 1958-1999 was calculated to be 2.25%. There was variation in susceptibility between seasons with spontaneous bursts of resistance interspersed with low levels of resistance. Frequency of resistance for H3N2 and H1N1 was very similar but there was no resistance detected within the H2N2 subtype. Within the H3N2 and H1N1 antigenic subtypes there was variation in susceptibility. It was hypothesised that this was due to the presence of quasispecies within the viruses containing populations of sensitive and resistant genotypes. Changes within the environment, allowing the resistant population a selective advantage over the sensitive, would have allowed the formation of a predominantly resistant population within a virus isolate. There was evidence to support the hypothesis that resistant viruses were not transmitted throughout the population, but within confined populations there was evidence to suggest that these events occurred. It was concluded that the emergence of resistant viruses within the UK was primarily a result of the mutating genome of influenza A viruses and less significantly from the use of amantadine to treat patients.

Surveillance of the drug susceptibility of isolates needs to be maintained. It is not fully known whether in the absence of selective drug pressure during multiple cycles of transmission resistant human viruses are capable of competing with wild-types (144). Results from this study would suggest not, but it is known that resistant viruses are equally virulent in animal models (350). This raises the possibility that if conditions were suitable, with sufficient selective pressure e.g. during an epidemic period where drug prophylaxis was widespread, resistant strains could outcompete wild-type strains to create an epidemic strain with a resistant genotype. It is interesting to note that unpublished findings from a recent study have demonstrated the persistence of amantadine-resistance within certain

swine influenza viruses (A. Hay, 2001; unpublished results). It is reported that in Europe, around 1986/87, an amantadine-resistant virus emerged in the swine population that has been maintained to present day. The exact reason for the emergence of this resistance is not known. The matrix genes from these resistant viruses have evolved along a separate lineage to their sensitive counterparts. The significance of these findings is twofold; firstly, it demonstrates the ability of an amantadine-resistant virus to be transmitted and maintained through an animal population for a long period of time without any selective pressure from drug. Secondly, pigs have been defined as a possible mixing vessel for the reassortment of influenza viruses from different animal species to form novel strains that have the ability to cause pandemic disease in humans. If the reassortment event occurred in a pig carrying an amantadine-resistant virus, there is the possibility that the novel virus might carry the matrix genes from the swine. This would result in a virus with novel antigenic properties that is able to infect a large number of hosts and also contain mutations conferring amantadine-resistance. This illustrates the importance of maintaining surveillance of amantadine-resistance in viruses from all host species

With the recent introduction of anti-neuraminidase drugs it is becoming evermore important to use population-based screening studies such as this to continually investigate the emergence of resistant viruses. The inevitable launch of additional drugs targeting influenza will exert an ever-increasing pressure on the virus to evolve and generate resistance. Protocols developed here can be used as templates for antiviral testing in the future in an attempt to monitor the spread of drug susceptibility.

Chapter 4

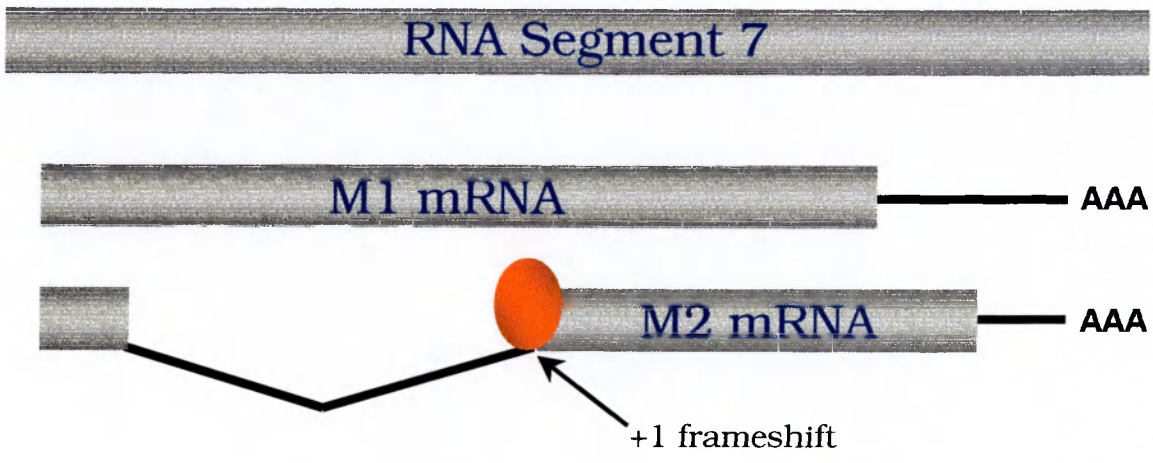
Genotypic Characterisation of Amantadine-Resistant Strains

4.1 Introduction

4.1.1 The Influenza A M2 Protein

The M2 protein of influenza A was implicated in having an essential role in the replication cycle of the virus during early studies of the antiviral drug amantadine (142). The influenza M2 protein is a small homotetramer consisting of 97 amino acids (204). It has three distinct regions; the extracellular, transmembrane and cytoplasmic domains, each of which contain 24, 19 and 54 amino acid residues, respectively (136). The M2 protein is greatly under-represented within the influenza virion when compared with other viral proteins e.g. M1, NP, HA1 or HA2, yet it is produced abundantly during infection and is expressed at the infected cell membrane in large quantities (411). M2 is coded by RNA segment seven of the influenza A genome. The segment contains two open reading frames (ORF), one encodes the M1 protein and the other ORF codes for the M2 protein in a +1 reading frame (204). The M2 mRNA contains a leader sequence of approximately 51 nucleotides that is covalently linked to the main body sequence (nucleotides 740-1011) and identical to the 5' end of the M1 mRNA (204) (Figure 4.1).

FIGURE 4.1 Coding strategies for M1 and M2 from influenza A gene segment 7



The orientation of the M2 protein within the virus membrane has been determined. The hydrophobic transmembrane domain of 19 amino acids (residues 25-43) anchors the protein to the virus membrane (206). The 54 amino acid COOH-terminal (C-terminal) domain is orientated on the cytoplasmic side of the membrane with the remaining NH_2-terminal (N-terminal) domain (24 residues) showing at the surface of the membrane with a minimum of 18 amino acids residues exposed (206). The actual function of the C-terminal domain of the M2 protein remains unknown although it has been shown to undergo post-translational modifications implying that it has some important role in the replication cycle (162, 348, 371). It has been suggested that the extracellular domain (N-terminal) of the M2 protein plays an important role in interacting with other membrane proteins, e.g. HA, NA and M1. Park et al (1998) constructed chimeric M2 mutants containing various combinations of the M2 domains and different regions from Sendai virus F protein (272). Results demonstrated that the only M2 chimeric mutant to be incorporated into influenza virions contained the M2 extracellular domain in combination with other regions of Sendai F protein. These findings suggested that the extracellular domain of M2 was important for its incorporation into virions (272).

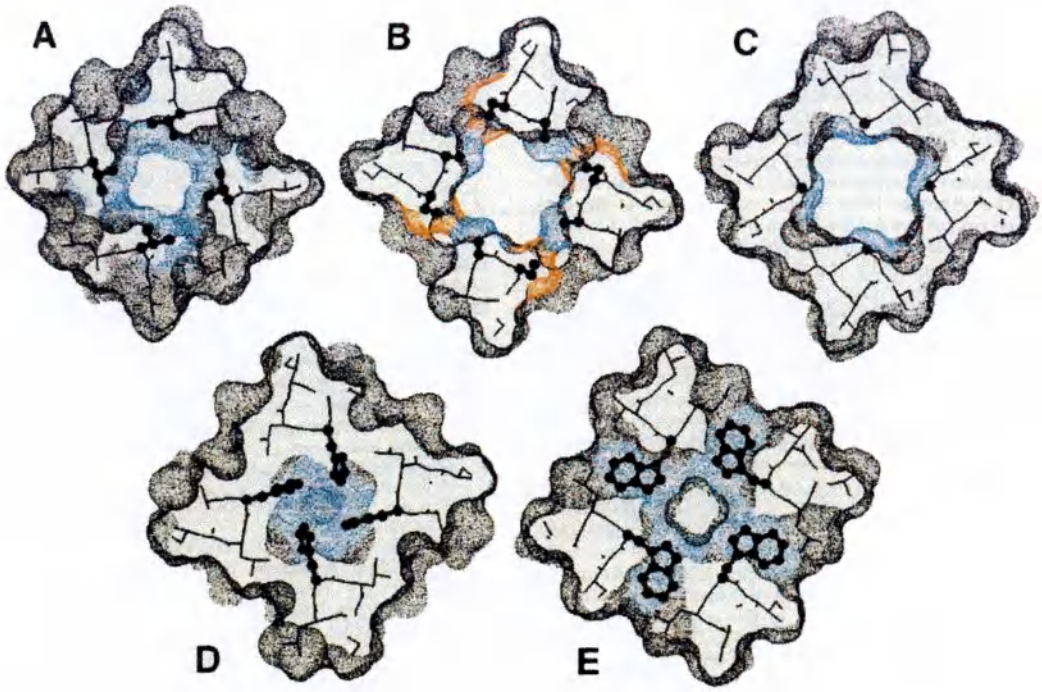
4.1.2 The Influenza A M2 Ion Channel

The M2 protein forms a membrane channel that permits the flow of protons (282). The ion channel theory was initially hypothesised by Sugrue *et al.* (1990) who investigated the control of pH during the influenza infectious cycle (346). Results from this early study provided evidence that M2 played a part in pH regulation by counteracting the acidic conditions of certain compartments of the exocytic pathway, in particular the TGN (346). It was proposed that M2 was able to regulate pH because it formed an ion channel within the virus membrane. Although there was no direct evidence at the time to support this theory, a further study by Sugrue and Hay (1991) presented data that indicated the M2 protein existed as two dimers, non-covalently held together by disulphide linkages at cysteine residues 17 and 19 (348); residue 17 was found to be conserved in all influenza A viruses (411). This proposed arrangement of two dimers forming a tetramer structure facilitated the ion channel theories; it created a structure that could resemble a channel with an inner pore lying within the virus membrane (348).

Two subsequent studies both independently provided evidence for the ion channel function of M2. Pinto *et al.* (1992) performed experiments where M2 mRNAs were microinjected into the oocytes of *Xenopus laevis* resulting in the expression of M2 protein (282). Membrane currents were measured using a two-electrode voltage-clamp procedure. Current readings across the membranes expressing M2 were taken in the presence and absence of amantadine, which was known to target the M2 protein. It was found that the M2 protein could be associated with ion channel activity and that the presence of amantadine attenuated the flow of current across the membranes expressing the protein. However, when the same procedure was repeated with oocytes expressing M2 proteins carrying mutations known to confer resistance to amantadine the drug did not block conductance (282). These data provided direct evidence that the M2 protein functioned as an ion channel and that the transmembrane domain of M2 formed the channel pore. Duff and Ashley (1992) corroborated these findings when they created a synthetic 25-residue peptide that corresponded to the predicted sequence of the transmembrane domain of the M2 protein (79). This peptide was incorporated into voltage-clamped planar lipid bilayers and a current passed across, again the results demonstrated the occurrence of proton conduction across the membrane. Similar studies using electrophysiological measurements of ionic current across different membranes expressing M2 including mammalian cells (46, 376), lipid vesicles and bilayers (320, 360) and in yeast (198) provided further evidence that M2 formed an ion channel and the transmembrane region of the protein formed the basis of the ion channel pore.

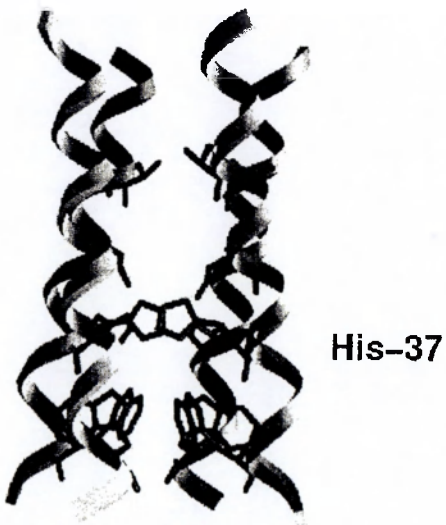
Previous studies have indicated the importance of certain amino acid residues within the pore and their effect on the structure and function of the channel. The histidine residue found at position 37 (His-37) has been established to have essential properties for controlling the "gating" of the channel (281, 321, 329, 416). The 3-dimentional structure of the inner pore of the channel has been predicted, revealing that His-37 occludes the channel, preventing the flow of protons (Figure 4.2) (281). Analysis of the structure of histidine (carrying a side-chain imidazole ring) suggests that within the tetramer structure, imidazole rings from two histidine molecules face each other and block the channel completely, resulting in the channel forming a closed state (Figure 4.3) (416).

FIGURE 4.2 Axial view of the predicted structure of the M2 ion channel



Panels A-E illustrate successive slices through the M2 ion channel in the direction of N-terminal to C-terminal. Amino acids thought to be essential for the function of the channel are characterised by ball-and-stick representations: (A) Val-27, (B) Ala-30, (C) Gly-34, (D) His-37 and (E) Tyr-41. It can be seen that His-37 (D) represents the only residue forming an occlusion within the channel. Taken from (281).

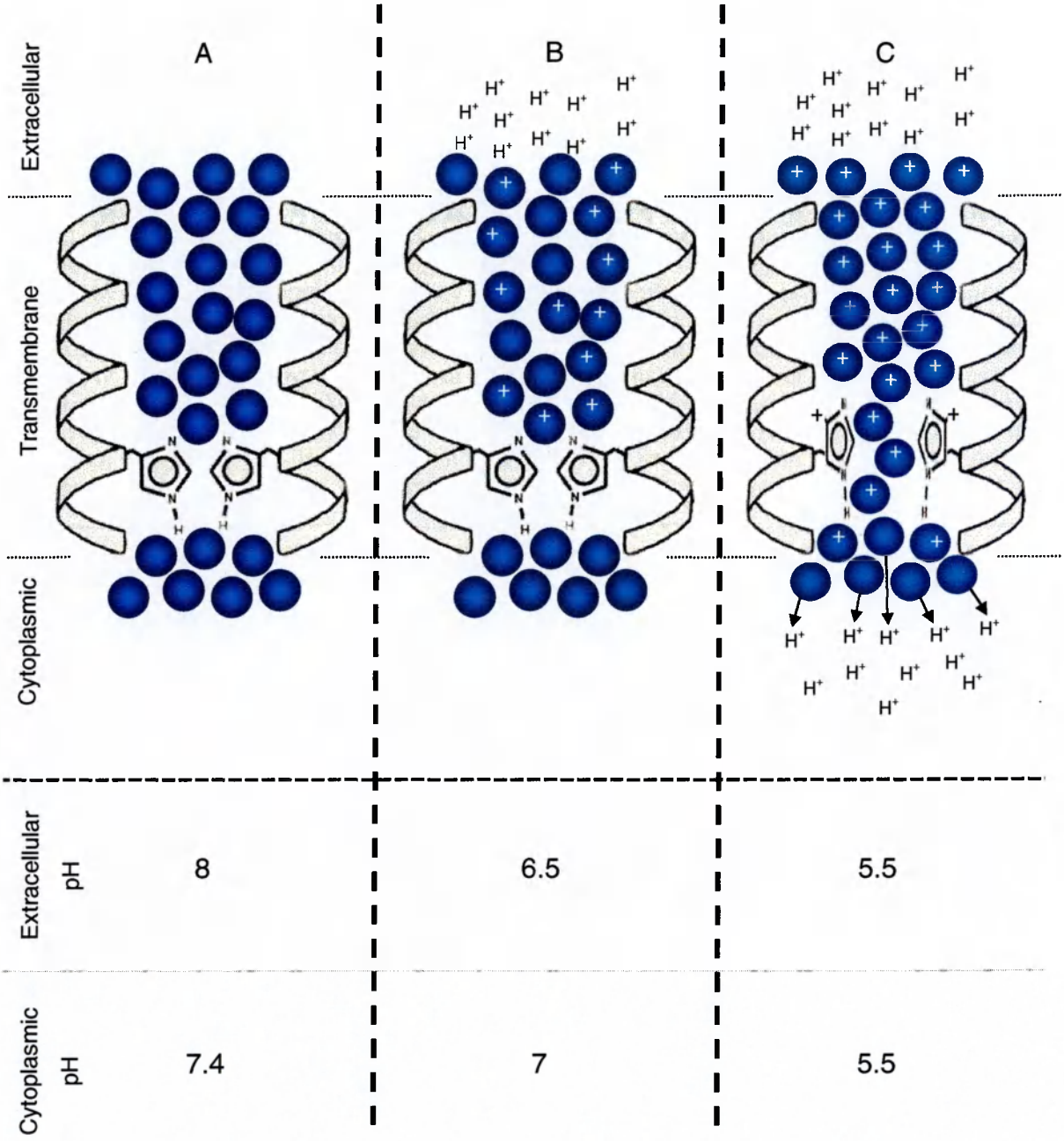
FIGURE 4.3 Representation of the side view of the M2 ion channel



The figure represents two helices of the M2 tetrad, with the N-terminal at top and C-terminal at bottom. The only major occlusion of the channel, His-37 is illustrated with the imidazole side-chain rings clearly visible. Taken from (329).

A mechanism for proton transportation within the M2 ion channel has been hypothesised (Figure 4.4). The ion channel is composed of four helices of M2 subunits that form a narrow pore that is occupied with water molecules (316). This channel of water spans the entire pore from the N-terminal mouth to the C-terminal end and consists of two to three parallel chains of "water wires" at any one time (99); this provides the basis for the transfer of protons along the channel. It is hypothesised that protons "hop" from one water molecule to the next and so move along the length of the ion channel from the N-terminal to C-terminal mouth (316). However, in order for the flow of protons to be complete through the entire channel, the pore must be in an open state i.e. the arrangement of the His-37 imidazole rings must be such that water molecules can span the entire channel. Within the closed channel, it has been proposed that the imidazole rings of His-37 residues remain in the lumen of the pore, thus the water molecules extend into the lumen of the pore from the N-terminal mouth to the point at which His-37 occludes the channel (316). Modelling studies have demonstrated that for the ion channel to open, protonation of the His-37 residues within the transmembrane tetramer structure must occur although it has been shown that only one His-37 residue needs to be protonated to facilitate the conduction of protons (281). This event results in a "tilting or flipping" of the ring structures of His-37, vacating the lumen of the pore and thereby facilitating a column of water to span the pore region fro the N-terminal to C-terminal mouth (Figure 4.4) (281, 416). Evidence for the importance of His-37 in the gating of the channel has been provided by studies where the His-37 residue was replaced with other amino acids (375). Resultant mutant ion channels, where the His-37 residues were replaced with residues that could not be protonated e.g. glycine or glutamate, were not activated when exposed to low pH (375). This demonstrated that the His-37 residue was vital in the gating of the channel and therefore, in the replication cycle of the virus.

FIGURE 4.4 Predicted water wire model for proton transduction through the M2 ion channel



The channel is viewed from the side with only His-37 showing; water molecules are represented by solid blue circles. (A) Pore in closed state; (B) lowered extracellular pH promotes protons (H^+) to "hop" along water wires occupying the lumen of the pore from N-terminal mouth to histidine occlusion; (C) protonation of the histidine causes imidazole rings to tilt or flip allowing column of water to fill pore from N-terminal to C-terminal mouth. Transfer of protons along length of pore promotes acidification of cytoplasmic domain. Predicted pH values of Rostock M2 ion channels expressed in *Xenopus laevis* ooccytes for the extracellular and cytoplasmic domains at different stages of ion channel activity are included below (324).

The M2 ion channel is found exclusively in the surface membranes of influenza A viruses. This is the fundamental reason why amantadine and rimantadine, anti-M2 protein drugs are effective only against infections caused by viruses of influenza A subtype. There is however, evidence to show that within influenza B and C viruses there are structural proteins analogous to the M2 ion channel that have similar functions. The NB protein is coded by influenza B gene segment six in an overlapping reading frame; NB is coded by the same mRNA as the NA surface glycoprotein (323). Studies to elucidate the structure and function of the NB protein were similar in nature to those described for the M2 ion channel (349, 394). The protein was found to be post-translationally modified by palmitoylation, the formation of disulphide linkages to form dimers and glycosylation of the N-terminal of the protein (38, 394). When the protein was expressed in *E. coli* and added to artificial lipid bilayers it was found that the protein produced ion channel activity. In comparison to M2, NB ion channels appeared to conduct other cations (Cl^-) in addition to protons, a finding that has not been fully explained (349). This finding was supported by results that demonstrated that the channel activity was blocked by the addition of an antibody targetlng the C-terminal end of the protein. Influenza B viruses are inhibited by amantadine *in vitro* when virus is exposed to concentrations approximately 100 times that required to inhibit influenza A viruses (10, 349). Although it has not been proven that amantadine at such high concentrations specifically inhibits the NB ion channel of influenza B viruses, it is probable that this is the case. Therefore, the available evidence indicates that the NB protein forms a cation-selective ion channel in influenza B viruses.

The influenza C M2 protein (CM2) is a 139 amino acid protein that is translated from a full length mRNA from segment six of the influenza C genome (164). Studies suggest that the CM2 protein is modified post-translation; it forms disulphide-linked dimers and trimers, and undergoes palmitoylation, a characteristic analogous to M2 (163, 164, 218). Another post-translational modification that CM2 shares in common with NB, but not M2, is glycosylation of the N-terminal domain of the protein (163, 394). The function of CM2 has not yet been determined and it remains to be seen whether it shares the ion channel activity of M2 and NB. A hydrophobic region within the protein has been identified that may serve as a transmembrane domain (276); considering this and the degree of homology between CM2 and the ion channels of influenza A and B it is

likely that CM2 does have a role in the control of pH within the replication cycle of influenza C viruses.

4.1.3 Post-Translational Modifications of M2

During the replication cycle of the influenza A virion the M2 protein undergoes several post-translational modifications. One modification is the addition of long chain fatty acids (acylation) to the M2 protein. An example of acylation is the addition of a palmitate moiety to the M2 protein (371). It has been determined that this event occurs during the transport of M2 to the cell surface and involves the majority of M2 molecules (347). The actual position of the palmitoylation has been determined through labelling of various influenza-infected cells with [^{3}H] palmitic acid. It was found that several equine viruses lacked an acyl group, which was correlated to the replacement of cysteine 50 with a phenylalanine residue (347). This demonstrated that the cysteine residue found at position 50 was the site of attachment for the palmitate fatty acid and therefore it was the cytoplasmic domain that was modified. The data also suggested that the linkage between the cysteine residue at position 50 and the palmitate moiety was a thioester linkage (347). Although the exact role of this modification was not clear, it was thought that the addition of the palmitate moiety to the cytoplasmic tail helped influence the stability of the M2 structure and possibly the formation of the M2 dimer (347).

Another post-translational modification that has been previously identified is the modification of the cytoplasmic tail by phosphorylation. There are five serine residues at positions 64, 71, 82, 89 and 93 found within the M2 protein that are possible targets for phosphorylation. Studies have shown that the serine residue residing at position 64 is the predominant phosphorylated residue within M2 (162). Serine residue 71 was not modified by the addition of phosphate, but the other serine residues at positions 82, 89 and 93 were all phosphorylated to a minor extent (162). The effect of phosphorylation on ion channel function was assessed and it was found that it had no discernable effect on membrane currents of M2 expressed in *Xenopus laevis* oocytes (162). Other hypotheses of how phosphorylation affects the virus have included the control of the intracellular transport of M2 and the incorporation of M2 into virus particles. However, recent experimental evidence has shown that unphosphorylated M2 is incorporated into

virus particles as efficiently as the phosphorylated form (358). This study also provided evidence that the phosphorylated cytoplasmic tail did not have any significant control on the intracellular transport of the M2 protein. Although the exact function of phosphorylation of the cytoplasmic tail is not fully understood, the conserved nature of serine residue 64 would suggest that phosphorylation at this position on M2 provides some evolutionary advantage to the virus (358).

The formation of disulphide bonds within the M2 tetramer structure represents another post-translational modification. The three possible sites for the disulphide linkages to form were identified as cysteine residues 17, 19 and 50. The cysteine residue at position 50 had previously been shown to be the target for covalent attachment of palmitate (347). It had been demonstrated that residues 17 and 19 were conserved in all influenza A viruses, which made them primary targets for the linkages (411). Evidence for the formation of disulphide linkages originated from ^{35}C-cysteine labelling of virus-infected cells and subsequent immunoprecipitation of virus peptides (161, 348). It was hypothesised that disulphide linkages held the two dimer structures together forming the tetramer which was the basis for the ion channel (348). Evidence has suggested that the formation of disulphide linkages has no effect on the function of M2 i.e. ion channel activity but the formation of disulphide linkages is thought to play a role in stabilising the M2 structure during stages of the replication cycle of the virus, although the exact role has not been fully characterised (162).

The incorporation of post-translational modifications within the C-terminal cytoplasmic tail region suggests that this region of the M2 protein would be essential for virus viability. Mutant viruses formed with truncated cytoplasmic tails have been shown to lose viability in correlation to the extent of truncation (45). Recombinant viruses containing M2 proteins lacking 5 and 10 residues from the cytoplasmic tail region could not be recovered by reverse genetics techniques, and viruses lacking only one residue from the C-terminal end had reduced growth potential in plaque assay. Similar experiments to determine the effect of C-terminal truncations on ion channel activity determined that several viruses carrying mutant M2 genes with certain truncations had reduced activity although some had activity that resembled the wild-type channel (359). It was hypothesised that the C-terminal of the M2 protein could have secondary structure, which in truncation mutants would might destabilise the protein and decrease the ion channel activity

of the virus. However, it has also been hypothesised that the M2 C-terminal domain may be important for sustaining interaction with other viral proteins; interactions with the M1 protein have previously been established (410, 411).

4.1.4 Function of the M2 Ion Channel

The M2 ion channel has been shown to have an essential role within the replication cycle of the virus. The two main functions of the channel are associated with its ability to transport protons and therefore regulate pH. Following the initial process of virus binding to the host cell receptors, the virion is internalised by receptor-mediated endocytosis (406). Cellular proton pumps within the endosomal membranes cause a gradual increase in the acidity of the internal environment within the endosome (136). The decrease in pH causes the HA to undergo a conformational change which promotes the fusion of viral and endosomal membranes (235). At the same time, the lowering of the internal endosomal pH stimulates the M2 ion channel and the pore "opens", allowing the flow of protons through it to occur (282). This process lowers the pH within the virion interior, which subsequently promotes the dissociation of the M1/RNP complex liberating the RNP for transport to the host cell nucleus (414).

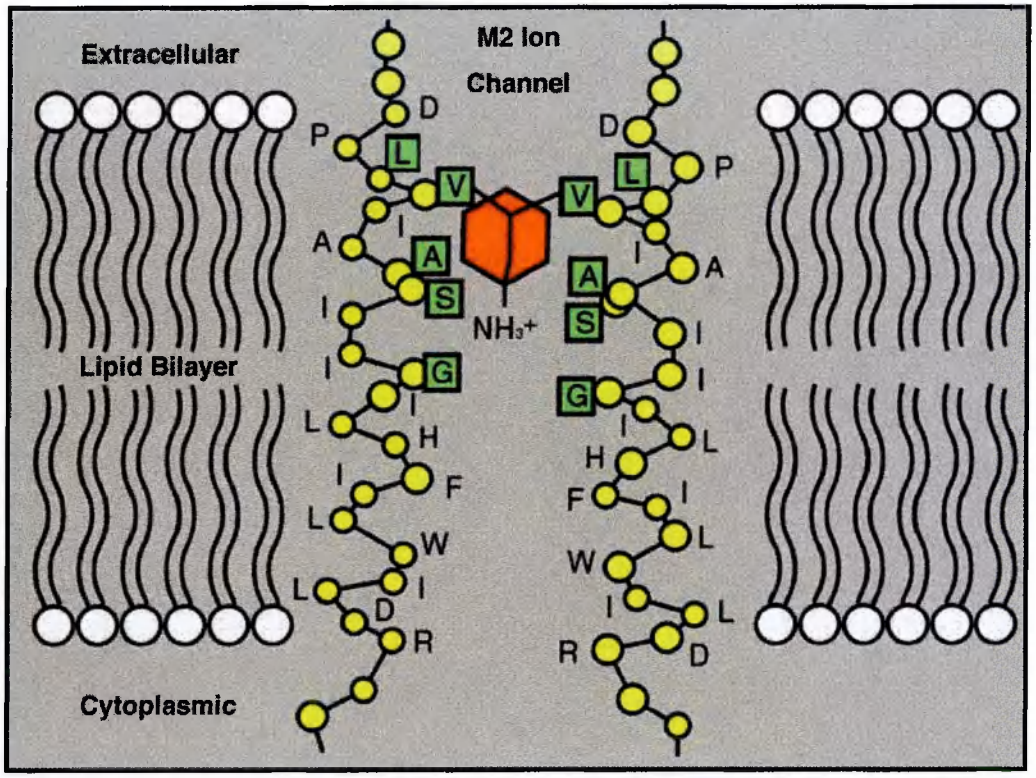
A second function of the M2 ion channel was discovered during studies of certain avian influenza A HA subtypes. Through studying the effect of amantadine on viral replication, it was found that the drug had an indirect affect on the structure of the avian HA (346). Certain avian HA species e.g. H7 and H5 contain a sequence of multi-basic amino acids at the cleavage site between HA1 and HA2 (182); this facilitates the proteolytic cleavage of the HA precursor HA0 into the HA1 and HA2 subunits in the TGN by the protease furin (341). The cleavage within the TGN renders the avian HA susceptible to structural conformational changes upon exposure to low pH (346, 392). In the late stages of the replication cycle, it was found that M2 was transported to the infected cell membrane via the TGN following synthesis in the endoplasmic reticulum (168). During the co-transport of the M2 and HA proteins from the site of synthesis in the endoplasmic reticulum to the infected cell membrane, the lumen of the TGN was maintained at an acidic pH by cell-mediated proton pumps. It was postulated that the M2 ion channel counteracted the acidity of the TGN by facilitating the flow of protons out of the

TGN via the ion channel thus increasing the pH. The effect of this pH regulation was that the pH-susceptible HA contained within the TGN was maintained in its native form; however, if the activity of the ion channel was suppressed, the HA was converted to a low pH form (346). The consequence of this transformation of native HA to a low pH form was not fully understood; the formation and distribution of budding particles from the cell surface were not significantly different between control and drug-treated cells but the release of particles was blocked (310). It was postulated that the presence of low pH HA inhibited a final stage in virus maturation e.g. pinching off of budding particles.

4.1.5 Molecular Basis for Mode of Action of Amantadine

Amantadine has been shown to have two concentration-dependent inhibitory actions on influenza A viruses (143). Early studies demonstrated that high concentrations inhibited early stages of infection and virus uncoating (39, 40, 143, 179), and low concentrations of drug indirectly affected the fusion activity of the viral haemagglutinin (143, 346). Therefore, the two inhibitory actions of amantadine correlate to the two defined functions of the M2 ion channel i.e. virus uncoating and HA maturation. Through the study of recombinant viruses composed of sensitive and resistant parental strains, the direct target of amantadine within the influenza A virion was initially shown to be the matrix protein (140, 224). The M1 and M2 proteins of resistant viruses were sequenced and the mutations conferring resistance were found to lie within a hydrophobic sequence of 19 amino acids that corresponded to the transmembrane domain of the M2 protein (142). Resistant viruses were determined as containing a single point mutation within M2 at one of five amino acid positions; 26, 27, 30, 31 or 34 (118, 142) but the mechanism behind the inhibition was unclear (Figure 4.5).

FIGURE 4.5 Diagrammatic representation of blockage of the M2 ion channel by amantadine



The figure represents the transmembrane domain of the M2 protein positioned within the lipid bilayer. Only two chains of the M2 tetramer are illustrated for clarity. Amino acid residues highlighted in green have been associated with amantadine-resistance. A molecule of amantadine (red) is shown lying within the ion channel pore. Adapted from (136).

There have also been reported cases of viruses carrying dual mutations within the M2 transmembrane domain that confer resistance (22, 118). Studies to assess amantadine-resistance in viruses recovered from both animals and humans have revealed a range of different substitutions that can occur at the five positions within the M2 transmembrane domain (Table 4.1). To date mutations in human viruses have been found at four amino acid positions, 26, 27, 30 and 31 (25, 147, 152, 166, 193).

TABLE 4.1 Amino acid substitutions in the M2 protein of amantadine-resistant viruses

Virus	M2 Transmembrane Amino Acid Position and Number									
	26		27		30		31		34	
A/New/York/83 (H3N2)			Val	→Ala	Ala	→Val	Ser	→Asn		
A/Virginia/88 (H3N2)			Val	→Ala	Ala	→Val →Thr	Ser	→Asn		
A/Shanghai/87 (H3N2)			Val	→Ala	Ala	→Val →Thr	Ser	→Asn		
A/USA/90 (H3N2)	Leu	→Phe					Ser	→Asn		
Ck/Penn/83 (H5N2)			Ile	→Ser →Thr	Ala	→Ser →Thr	Ser	→Asn		
Ck/Germany/27 (H7N7)			Val	→Ala →Gly →Asp	Ala	→Thr →Pro	Ser	→Asn	Gly	→Glu

Only amino acid residues within the M2 transmembrane region known to be important in amantadine-resistance are shown. Under each residue, the sensitive wild-type is shown on the left of the arrow, and the resistant mutants on the right. All resistant viruses were isolated *in vivo* except Ck/Germany/27 that were isolated by plaque selection in the presence of amantadine. Taken from (137).

It is apparent that resistant viruses isolated from humans during or following therapeutic treatment with amantadine or rimantadine predominantly carry a Ser to Asp substitution at position 31 (Ser31Asn) (90, 144, 147, 166, 417). It has been postulated that this mutation may provide the best fitness to viruses carrying it with regard to transmission, and therefore survival. Studies of resistant viruses within the avian model have revealed that mutations can be found at all five positions but several mutations reported are different from ones emerging in resistant human viruses e.g. Leu26His and Val27Asp (118). Within the avian model, resistant

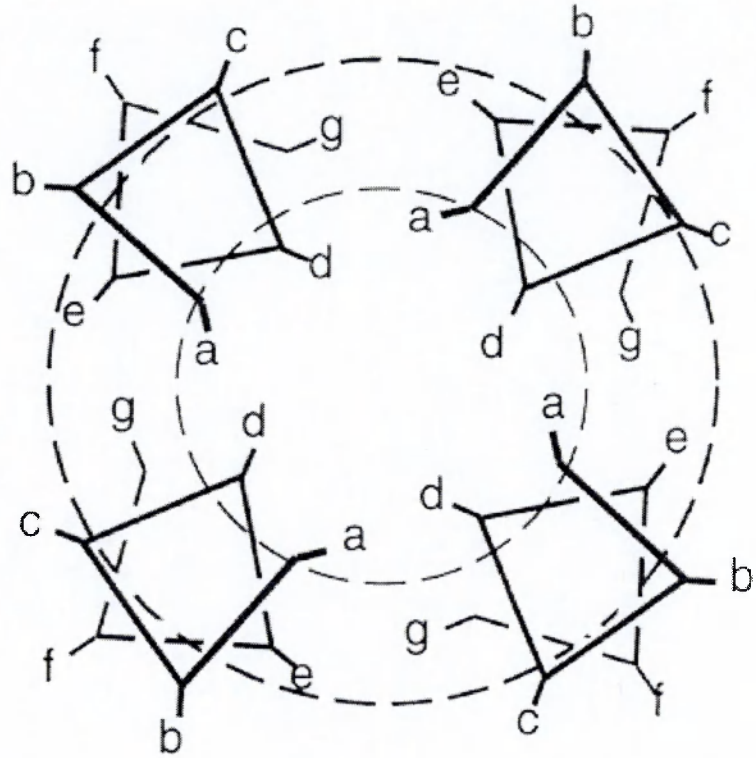
mutations appear to be dependent on the virus strain, e.g. in viruses of Rostock strain (H7N1), mutations were found to be exclusively at position 27 but in the Weybridge strain (H7N7) viruses mutations were predominantly found at position 34 (142). Therefore, it appears that the occurrence of mutations within influenza A viruses are dependent on the host species and the virus strain. There are other potential factors affecting selection of different resistant genotypes that are not yet fully understood.

The occurrence of mutations within the transmembrane region of the M2 ion channel can influence different functional aspects of the virus. It has been reported that mutations at different positions within M2 have increased or decreased the activity of the protein resulting in corresponding changes in viral infectivity and growth (118). This was observed in avian strains of influenza where amino acid substitutions at positions 26, 30, 31 and 34 caused an increase in the expression of the low-pH form of HA as a result of suppression of the M2 ion channel activity. In contrast, viruses that carried a substitution at position 27 (Ile to Thr or Ser) were shown to have greater growth and infectivity compared to wild-type virus indicating a possible increase in M2 activity (118). These results are interesting as they raise the question of whether a resistant mutant could gain a selective advantage over sensitive wild-type strains by acquiring amantadine-resistance at certain positions in the M2 transmembrane domain. To date, studies concerning this aspect of resistance have been limited to avian influenza; the implication for the virulence and transmissibility within human strains and therefore the general population requires further investigation.

Molecular dynamics studies have revealed the predicted orientation of the M2 ion channel and the pore (281, 329). This has enabled the mapping of the location of important residues, which form the face of the lumen of the channel pore. Data from such studies may explain why certain residues within the channel have more structural significance, and are associated with amantadine-resistance. Modelling has revealed that certain residues known to confer amantadine-resistance were located on the lumen face of the M2 ion channel pore (Figure 4.6). It has been hypothesised that the positioning of the residues facilitate the interaction of the respective amino acids with amantadine molecules and therefore represent positions where amantadine can interact and block the channel. Mutations at any of the five positions have been shown to confer resistance and it is thought that

substitutions of the residues may result in changes in the channel structure and neutralise the binding of amantadine within the pore region.

FIGURE 4.6 Helical wheel diagram representing one heptad of the four-helical bundle of the M2 protein



The dashed line represent the boundaries between three different regions; the central pore of the ion channel, the middle region where the helix-helix interface lies, and the outer region representing the lipid bilayer. The transmembrane sequence below (residues 25-43) illustrates the heptad repeat of residues *a* and *d*, which corresponds to Val-27, Ala-30, Gly-34 and Tyr-41. Adapted from (281).

4.2 Aims

The aim of this work was to characterise the amantadine-resistant viruses that had been selected through amantadine susceptibility screening performed by EIA and plaque reduction assay. It was hoped that this would reveal the relative frequency of M2 mutations in human populations and influenza subtypes. The objectives were to determine the underlying molecular changes conferring resistance in the viruses and therefore their resistance genotype. Primarily, this involved sequencing M2 and analysing the transmembrane domain, the region that was known to be the target of amantadine and where substitutions conferring resistance occurred. With the resulting data, amino acid sequences were aligned to identify changes within M2 and also to determine the presence of any changes within M1 that might have contributed to changes in virus function. A selection of amantadine-sensitive viruses was chosen from the susceptibility screening and M2 sequenced to determine whether there were any significant amino acid substitutions within the proteins of the sensitive strains.

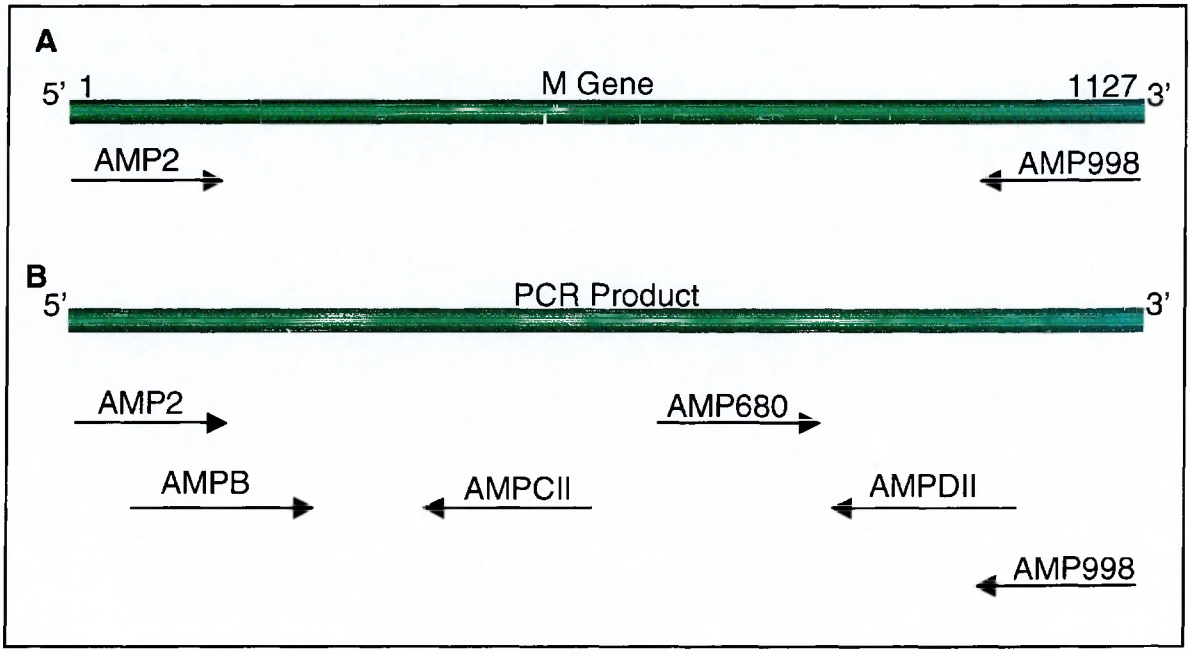
Understanding the consequence of amino acid changes within M2 on the biological properties of the viruses was important because the matrix genes have been implicated in controlling virus growth. It was hoped that comparisons of sequence data and phenotypic results from Chapter 3 would reveal whether specific mutations within M2 conferred any selective advantage to the viruses.

4.3 Results

4.3.1 PCR Amplification of Resistant Strains

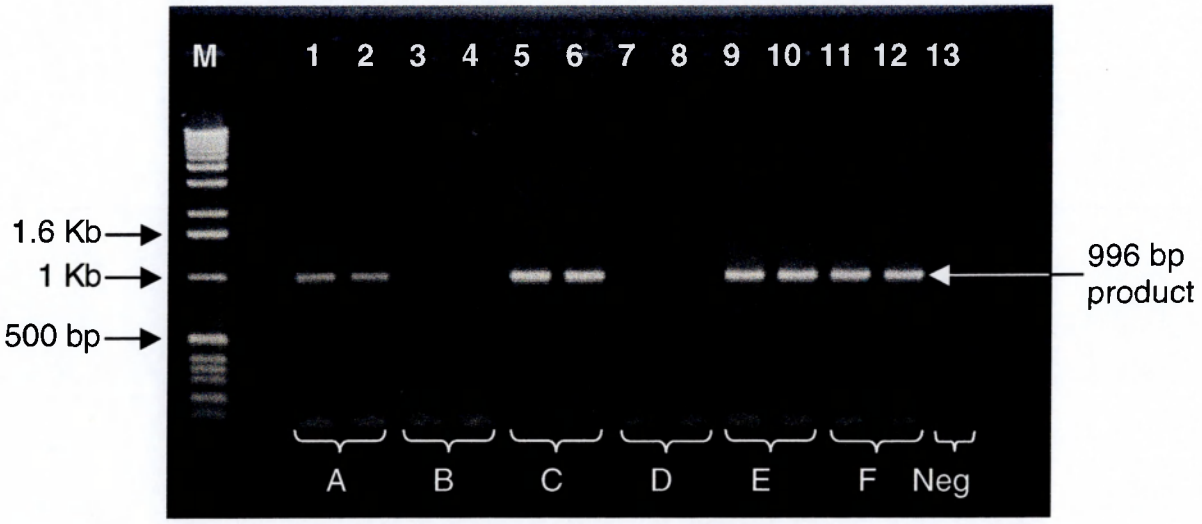
RNA was extracted from tissue culture fluid containing the resistant viruses, reverse transcribed into cDNA and amplified by PCR as described in Materials and Methods (Chapter 2, page 45). The PCR primers used to amplify the entire M gene were designated AMP2 and AMP998 (Figure 4.7). PCR amplicons were detected by agarose gel electrophoresis and ethidium bromide staining. The correct size for bands corresponding to the amplified M gene was 996 base pairs (Figure 4.8). The presence of a band of this size indicated that the M gene had been successfully amplified from the specimen; the PCR product was then stored for further analysis.

FIGURE 4.7 Oligonucleotide primer positions for (A) PCR amplification and (B) sequencing of gene segment seven



Green bars represent the target gene and PCR amplicon. Primers used for PCR amplification are designated with arrows and represent the approximate position of amplification.

FIGURE 4.8 A typical gel image produced by M gene PCR amplification



Gel electrophoresis of samples A-F. Lanes 1&2, 3&4, 5&6, 7&8, 9&10, 11&12 were replicates of resistant samples A-F. Lane 13 contained a negative control consisting of virus transport medium. Samples A (lanes 1&2), C (lanes 5&6), E (lanes 9&10) and F (lanes 11&12) were all positive for influenza M gene; samples B (lanes 3&4) and D were negative for M gene. M – DNA molecular weight marker.

In cases where the PCR did not amplify the M gene and the 996 base pair product could not be visualised on the agarose gel, the original virus isolate was inoculated into tubes separately seeded with confluent MDCK and RMK mammalian monolayers. After incubation for seven days at 37°C the tissue culture fluid was re-extracted and the PCR repeated.

From EIA and plaque reduction testing, 48 viruses were selected as phenotypically resistant to amantadine. PCR amplification was performed on 48/48 resistant viruses; results showed that 47/48 contained a product of 996 base pairs corresponding to the M gene. The only virus from which genetic material could not be extracted was A/ENG/424/98; even after attempted re-amplification in tissue culture there was still no positive PCR result. Therefore, this virus could not be processed any further and thus the total number of resistant viruses that were analysed by genotypic methods was 47.

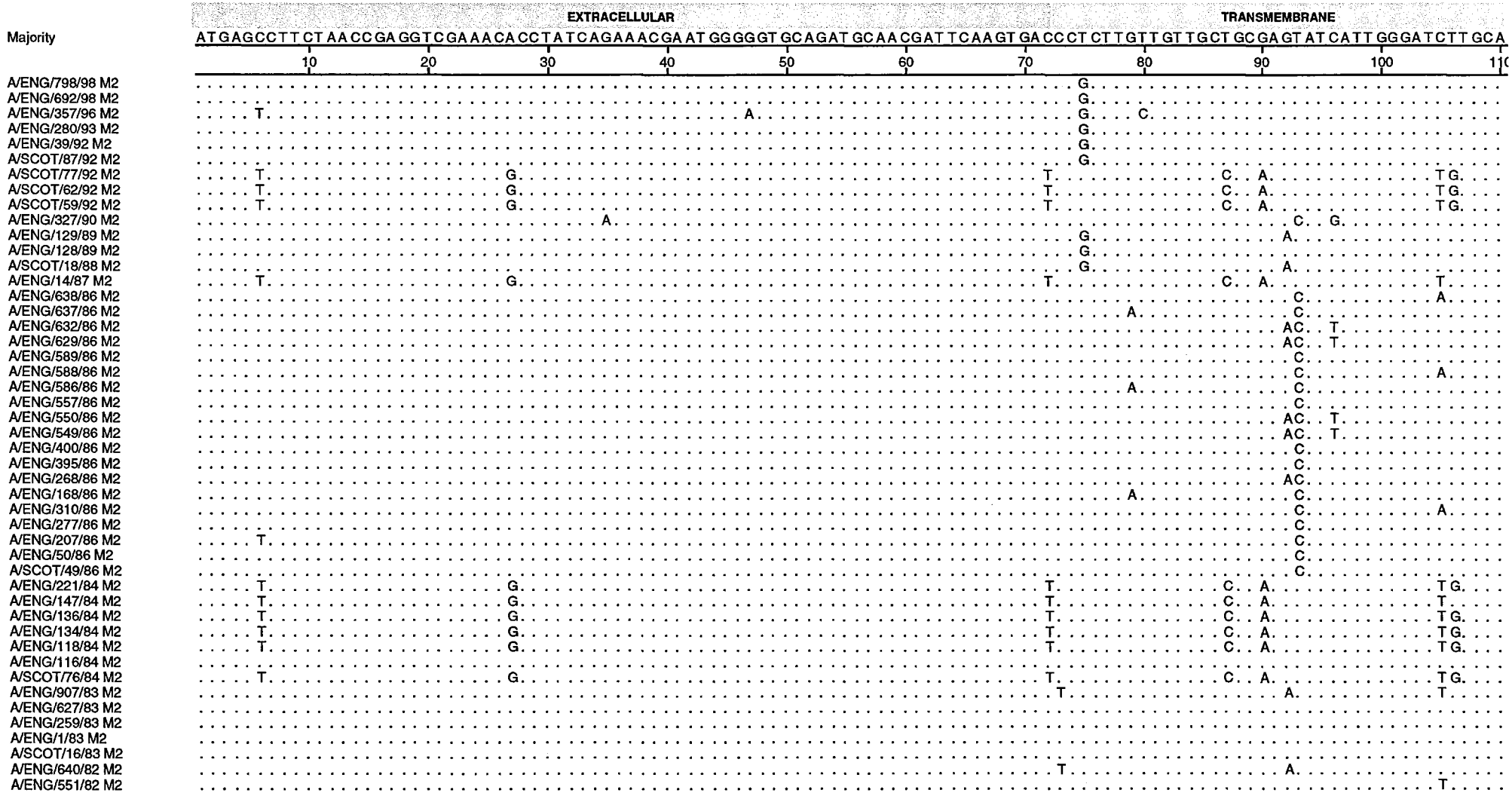
4.3.2 M2 Genes Originating from Resistant viruses

Automated sequencing was performed on M gene PCR products of resistant viruses as described in Materials and Methods (section 2.13). The positions of the primers used in the sequencing reactions are illustrated in Figure 4.7. The majority of viruses were sequenced using an Applied Biosystems 373 automated sequencer although a proportion of viruses were sent to Durham University DNA sequencing service to be analysed.

Raw data extracted from the Applied Biosystems 373 sequencer was analysed using the sequence analysis software package, SeqEd (Applied Biosystems). Nucleotide sequences from each primer were checked, bases were called, and then the sequence aligned to produce a nucleotide sequence corresponding to the whole matrix gene. In order to produce a nucleotide sequence for the entire M2 gene, two sections of the matrix sequence had to be cut and then spliced. Nucleotides 26-51 and 740-1004 were spliced together in EditSeq (DNAStar) to form a sequence 291 bases in length for each virus; this corresponded to the known size of M2 (204). Sequences were then imported into MegAlign (DNAStar) where nucleotide and amino acid alignments were created. Changes within the nucleotide or amino acids were compared to the majority sequence calculated by MegAlign.

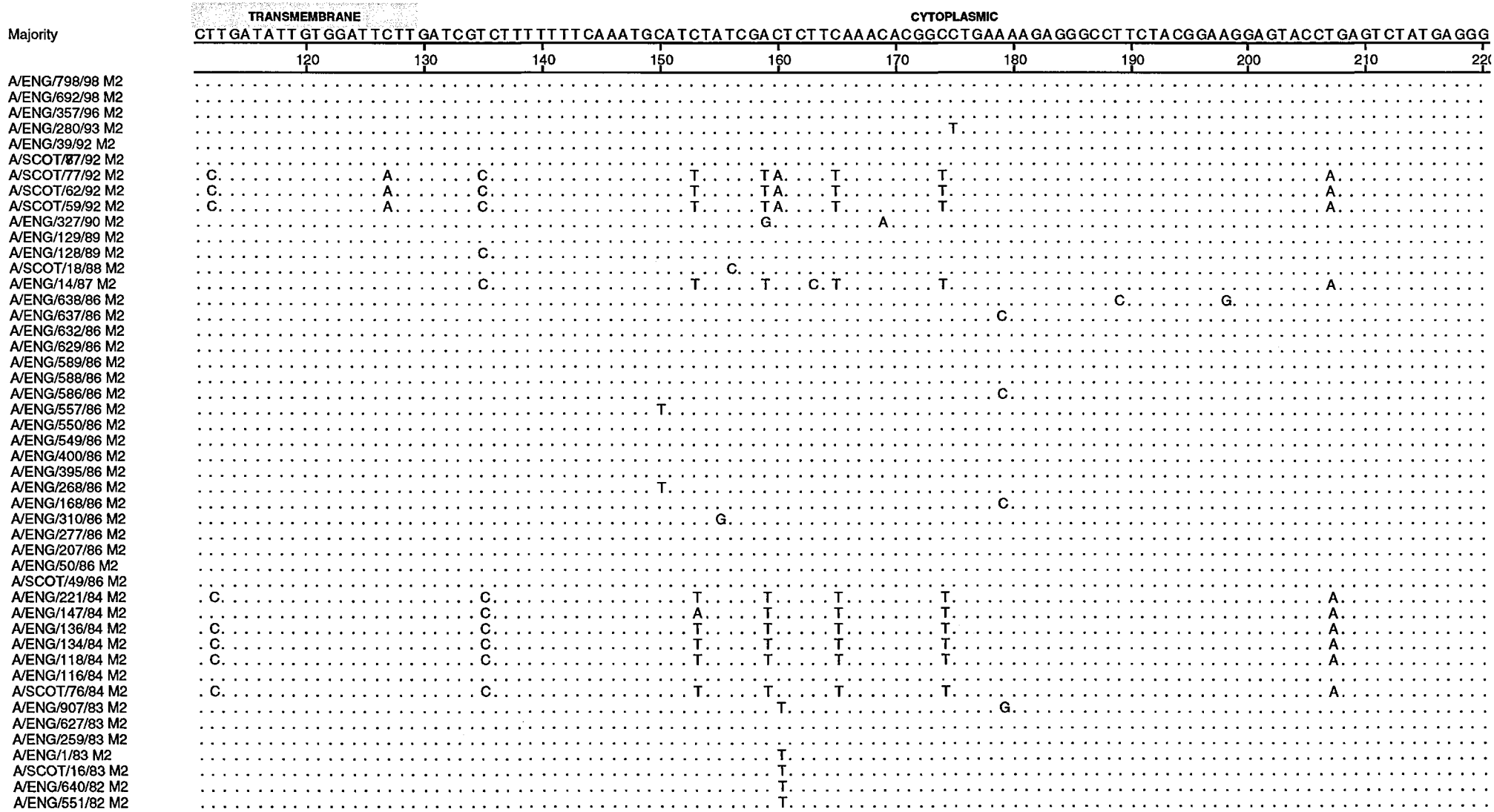
Initially, nucleotide sequences for M2 were prepared for all of the resistant viruses and aligned against the majority sequence calculated by MegAlign (Figure 4.9). From initial analysis of the nucleotide alignments it was apparent that there were numerous nucleotide substitutions occurring in the resistant viruses. There were only two viruses that had sequences homologous to the majority, A/ENG/627/83 and A/ENG/259/83. The sequences were divided into the three known functional domains of M2 to determine whether the substitutions were more prevalent in certain regions. The extracellular domain (residues 1-72) contained relatively few sites where nucleotide substitutions occurred. At residues 6, 27 and 72 there were 12/47, 10/47 and 10/47 viruses that had mutations from C→T, A→G and C→T respectively; 10/12 viruses that carried a mutation at one of these three positions carried all three mutations. In addition, there were two viruses (A/ENG/357/96 and A/ENG/327/90) that carried a single G→A mutation at positions 35 and 47 in the extracellular domain.

FIGURE 4.9 Alignment of M2 nucleotide sequences from phenotypically resistant viruses



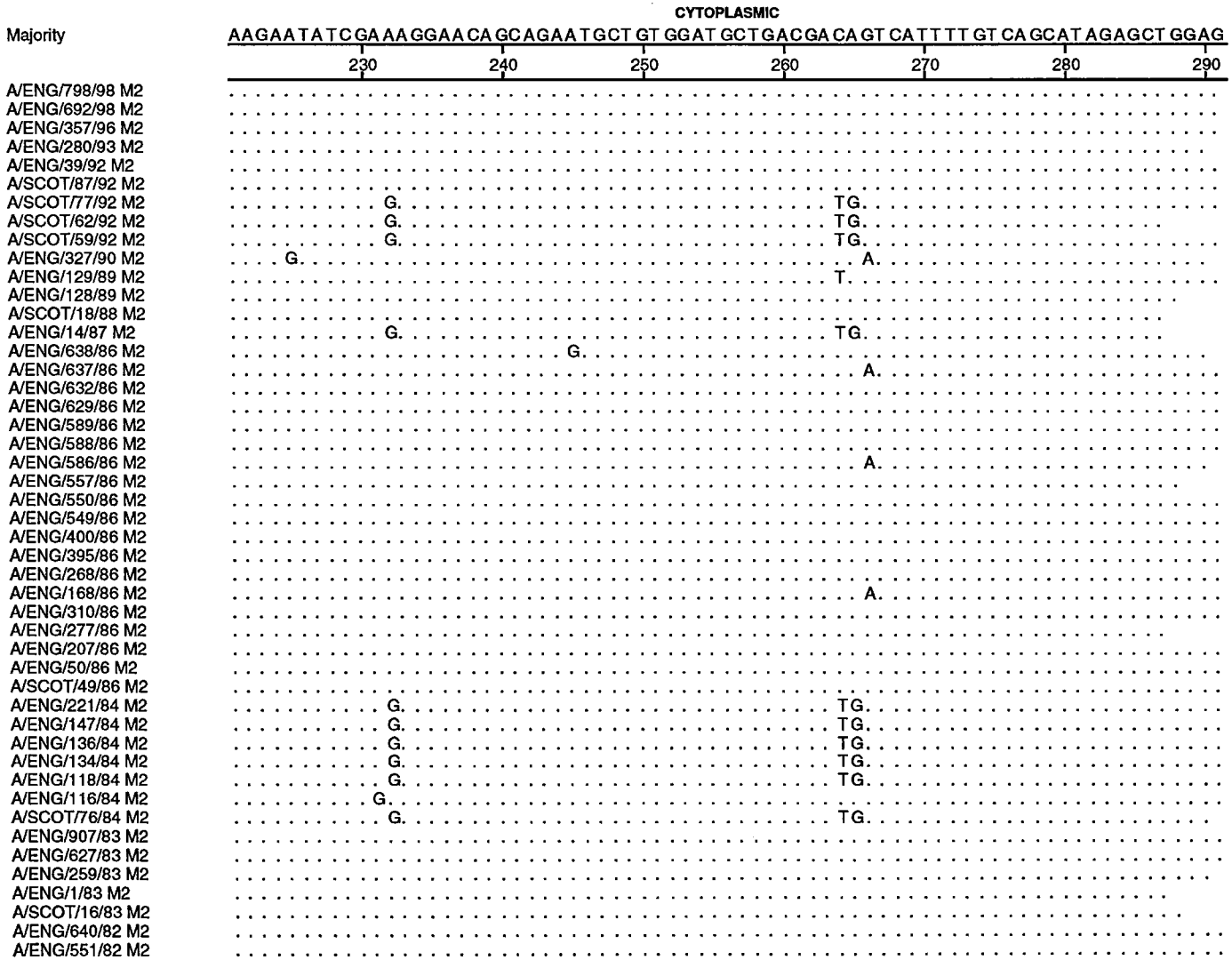
Resistant viruses are ordered chronologically and the nucleotides aligned to a majority sequence calculated by Megalign.

FIGURE 4.9 Alignment of M2 nucleotide sequences from phenotypically resistant viruses (continued)



Resistant viruses are ordered chronologically and the nucleotides aligned to a majority sequence calculated by Megalign.

FIGURE 4. 9 Alignment of M2 nucleotide sequences from phenotypically resistant viruses (continued)



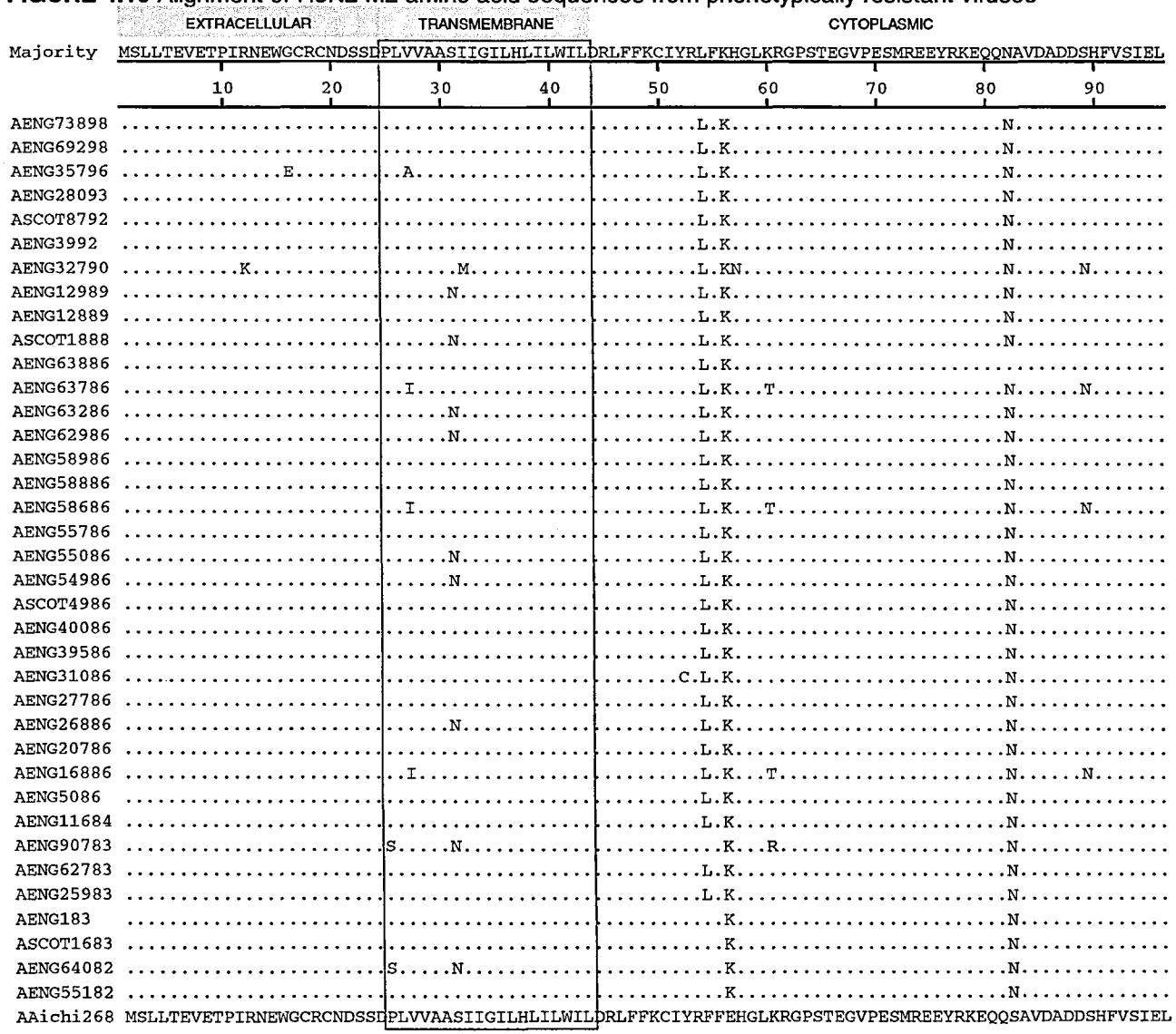
Resistant viruses are ordered chronologically and the nucleotides aligned to a majority sequence calculated by Megalign.

In order to determine whether the substitutions found within the extracellular domain were silent, or coding for amino acid changes, an amino acid alignment was prepared for H3N2 and H1N1 viruses using MegAlign (Figures 4.10 and 4.11). From these alignments it was clear that there were only two amino acid substitutions that had occurred, both within H3N2 viruses. One mutation was a Gly→Glu at position 16 (Gly16Glu) and the other an Arg12Lys. It was deduced that these amino acid substitutions had been caused by the single G→A nucleotide substitution at residues 35 and 47 (Figure 4.9). Therefore, the remaining nucleotide substitutions within the extracellular domain were all synonymous (silent) suggesting that non-synonymous changes causing amino acid mutations within this domain were detrimental to the function of the extracellular region.

The nucleotide and amino acid alignments for the transmembrane domain (nucleotide residues 73-129) of M2 were analysed (Figures 4.9 to 4.11). In comparison, there were significantly more mutations in the transmembrane region than the extracellular region. Analysis of both nucleotide and amino acid substitutions revealed that approximately 26% of the nucleotide changes found within the transmembrane region were non-synonymous i.e. coding for an amino acid change. This figure was relatively high when compared to the percentage of non-synonymous nucleotide changes within the extracellular domain (6%). When the non-synonymous changes within the transmembrane region were analysed, it was found that they comprised of 12 transitions and 15 transversions. The amino acid changes within the transmembrane domain were analysed and compared to previous studies where amantadine-resistant viruses had been characterised. From a total of 47 viruses that had been sequenced, 22 carried a mutation within the transmembrane region. The most frequent mutations found were Ser31Asn (9/22) and Leu36Val (8/22). The remaining viruses had Val27Ala (1/22), Val27Ile (3/22) and Ile32Met (1/22) substitutions. In addition, several viruses carried dual mutations within the transmembrane region; three viruses carrying a Leu36Val also had a Leu43Ile mutation. Similarly, two viruses with Ser31Asn had an additional Pro25Ser. These results obtained from sequencing M2 confirmed previous findings that the Ser31Asn mutation was commonly found in resistant H3N2 viruses isolated from humans. The Val27Ala mutation that was found in A/ENG/357/96 had also previously been described in human resistant strains. The remaining viruses carrying mutations in the transmembrane domain were

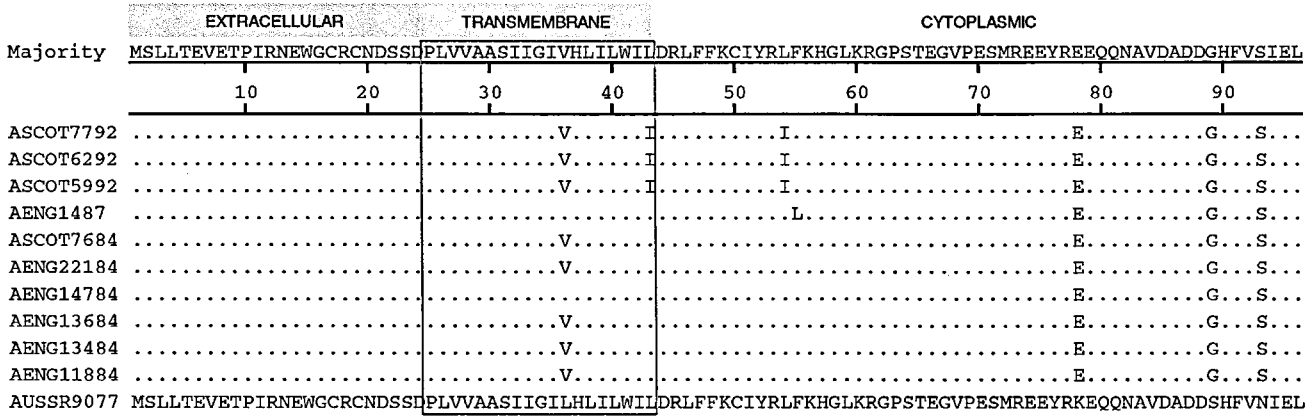
interesting because there had been no previous documentation of their occurrence in human resistant viruses. The Leu36Val mutation was discovered in eight H1N1 viruses originating from the 1983/84 and 1991/92 seasons. Three viruses from 1991/92 carried an additional Leu43Ile mutation. Four viruses were found to have mutations at position 27; one Val27Ala and three Val27Ile substitutions. The Val27Ala had been previously found in human viruses but the Val27Ile substitutions could not be found in the M2 transmembrane sequences of any human viruses. The M2 transmembrane mutations are summarised in Table 4.2.

FIGURE 4.10 Alignment of H3N2 M2 amino acid sequences from phenotypically resistant viruses



Resistant viruses are ordered chronologically. The H3N2 virus sequences are aligned to the prototype H3N2 virus A/Aichi/2/68. The transmembrane domain is boxed in red.

FIGURE 4.11 Alignment of H1N1 M2 amino acid sequences from phenotypically resistant viruses



Resistant viruses are ordered chronologically. The H1N1 virus sequences are aligned to the prototype H1N1 virus A/USSR/90/77. The transmembrane domain is boxed in red.

TABLE 4.2 Summary of the amino acid sequences of viruses containing mutations within the M2 transmembrane domain

VIRUS	SEASON	SUBTYPE	M2 TRANSMEMBRANE SEQUENCE (AMINO ACID POSITION AND NUMBER)																	
			25	26	27	28	29	30	31	32	33	34	35	36	37	38	29	40	41	42	43
			Pro	Leu	Val	Val	Ala	Ala	Ser	Ile	Ile	Gly	Ile	Leu	His	Leu	Ile	Leu	Tyr	Ile	Leu
A/ENG/357/96	96/97	H3N2	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A/ENG/637/86	85/86	H3N2	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A/ENG/580/86	85/86	H3N2	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A/ENG/168/86	85/86	H3N2	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A/ENG/129/89	88/89	H3N2	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-
A/SCOT/18/88	88/89	H3N2	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-
A/ENG/632/86	85/86	H3N2	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-
A/ENG/629/86	85/86	H3N2	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-
A/ENG/550/86	85/86	H3N2	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-
A/ENG/549/86	85/86	H3N2	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-
A/ENG/268/86	85/86	H3N2	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-
A/ENG/907/83	83/84	H3N2	S	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-
A/ENG/640/82	81/82	H3N2	S	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-
A/ENG/327/90	89/90	H3N2	-	-	-	-	-	-	-	M	-	-	-	-	-	-	-	-	-	-	-
A/SCOT/77/92	91/92	H1N1	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	I
A/SCOT/62/92	91/92	H1N1	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	I
A/SCOT/59/92	91/92	H1N1	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	I
A/ENG/221/84	83/84	H1N1	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-
A/ENG/136/84	83/84	H1N1	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-
A/ENG/134/86	83/84	H1N1	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-
A/ENG/118/84	83/84	H1N1	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-
A/SCOT/76/84	83/84	H1N1	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-

Substitutions at amino acid positions highlighted in red are known to confer amantadine-resistance (118, 142). Viruses are categorised depending on the amino acid mutation contained within the transmembrane region.

The nucleotide and amino acid sequences of the cytoplasmic domain (nucleotide residues 130-291) were analysed; between the 47 resistant viruses sequenced there were 121 nucleotide changes observed (Figure 4.9). In relation to the size of the domain (162 nucleotides) mutations occurred at a frequency of approximately 1.6 nucleotide changes per site per virus compared to 1 and 3.9 for the extracellular and transmembrane domains respectively. This demonstrated that the cytoplasmic region was more conserved than the transmembrane domain but not to the degree of conservation shown by the extracellular domain. Approximately 33% of the nucleotide changes were non-synonymous. This indicated that the cytoplasmic domain had the highest rate of non-synonymous mutations throughout the three functional domains of M2. All of the molecular changes observed in the domains of M2 of the resistant viruses are summarised in Table 4.3.

TABLE 4.3 Summary of M2 sequence mutations within 47 resistant viruses analysed

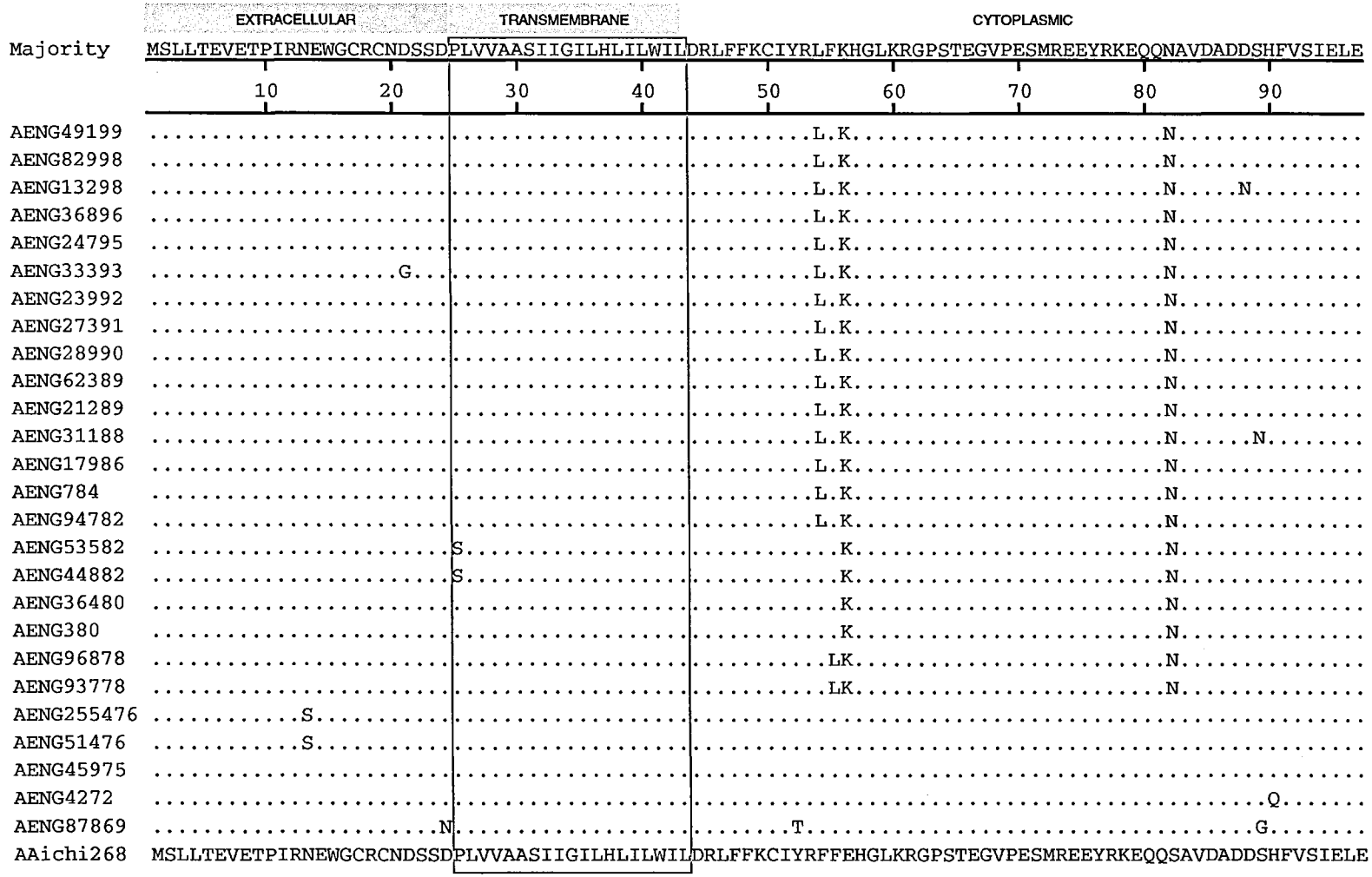
	M2 Domain (No. Nucleotides)		
	Extracellular (72)	Transmembrane (57)	Cytoplasmic (162)
Nt changes	34	103	121
Synon (Ts/Tv)	32 (32/0)	76 (64/12)	81 (60/12)
Non-synon (Ts/Tv)	2 (2/0)	27 (12/15)	40 (33/7)
No. nt changes/ site/virus	0.01	0.03	0.02

Nt – nucleotide; Synon – synonymous; Non-synon – non-synonymous; Ts – transition (purine to purine / pyramidine to pyramidine); Tv – transversion (purine to pyramidine / pyramidine to purine).

4.3.3 M2 Genes Originating from Sensitive Viruses

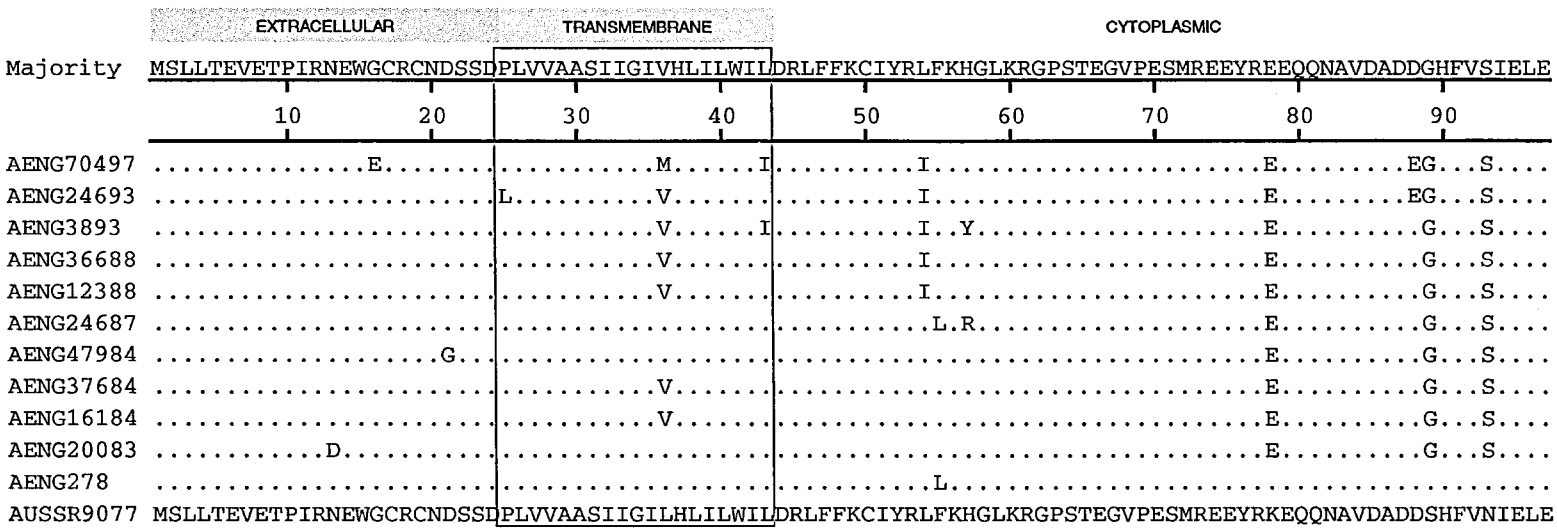
In order to determine whether the mutations found within the M2 gene were exclusive to resistant viruses, a selection of viruses that were sensitive to amantadine by EIA were sequenced. The amino acid sequences of H3N2 and H1N1 were aligned separately against A/Aichi/2/68 and A/USSR/90/77 respectively (Figures 4.12 and 4.13). The H3N2 alignment revealed that out of 26 sensitive viruses sequenced, two contained mutations within the transmembrane region (Figure 4.12). Eleven sensitive H1N1 viruses were sequenced and there were seven viruses carrying M2 transmembrane mutations, three viruses each contained two separate mutations (Figure 4.13). The amino acids changes found within all viruses were at positions that had not previously been associated with amantadine-resistance but several shared mutations with viruses that had been found to be phenotypically resistant in this present study. Seven H1N1 viruses carried an amino acid substitution at position 36; the majority (6/7) contained a Leu36Val mutation, identical to the mutation found within the eight resistant viruses (Figure 4.11 and Table 4.2). The other mutation at this position was a Leu36Met; this mutation had not been found in the resistant strains. There were other mutations found in the transmembrane region of the sensitive strains that were in common with mutations found in the resistant strains. Two viruses carried a Pro25Ser and two carried a Leu43Ile, both mutations had been found in the resistant strains (Figures 4.10 and 4.11).

FIGURE 4.12 Alignment of H3N2 M2 amino acid sequences from phenotypically sensitive viruses



Sensitive viruses are ordered chronologically. The H3N2 virus sequences are aligned to the prototype H3N2 virus A/Aichi/2/68. The transmembrane domain is boxed in red.

FIGURE 4.13 Alignment of H1N1 M2 amino acid sequences from phenotypically sensitive viruses

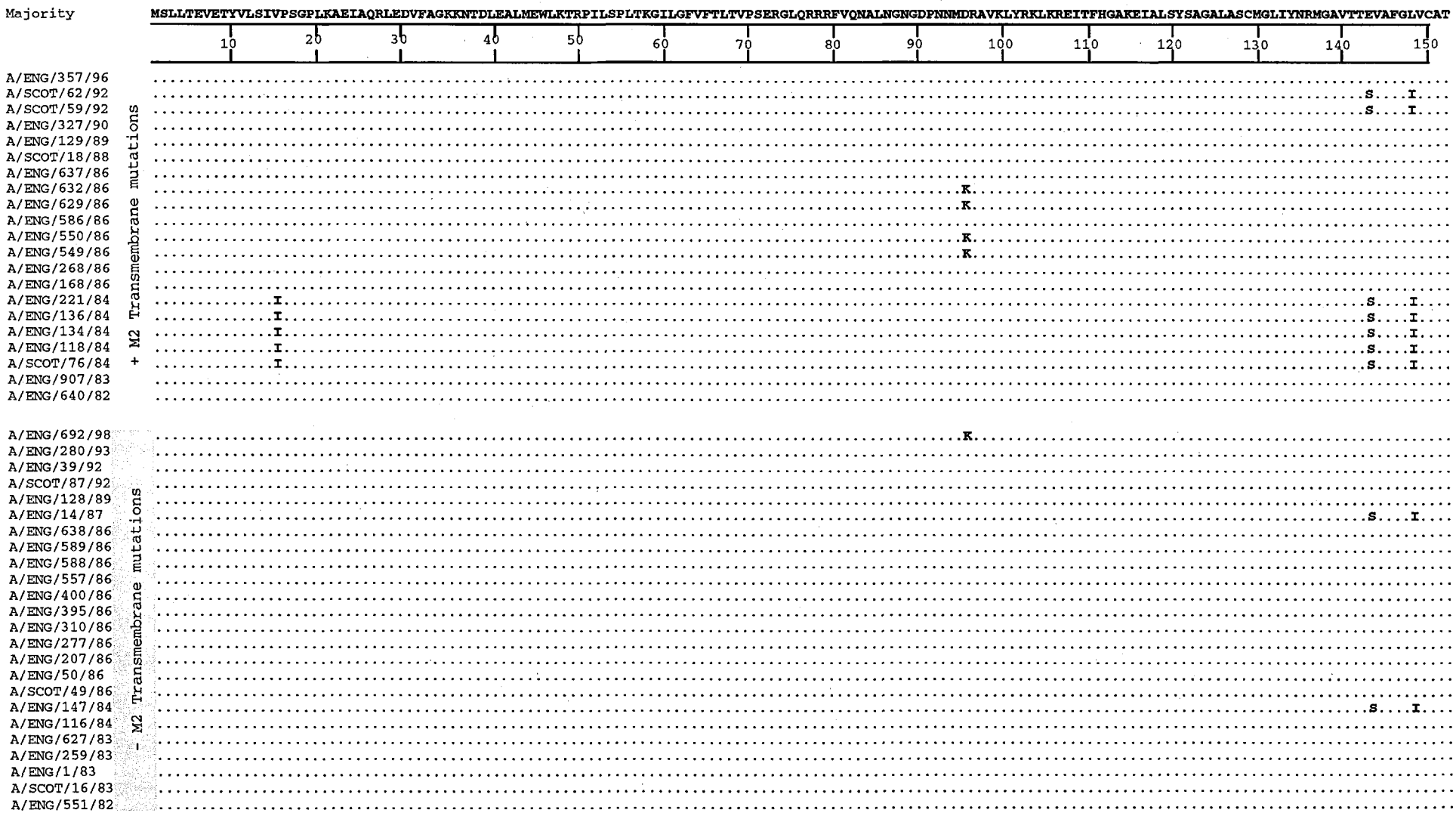


Sensitive viruses are ordered chronologically. The H1N1 virus sequences are aligned to the prototype H1N1 virus A/USSR/90/77. The transmembrane domain is boxed in red.

4.3.4 M1 Genes Originating from Resistant Viruses

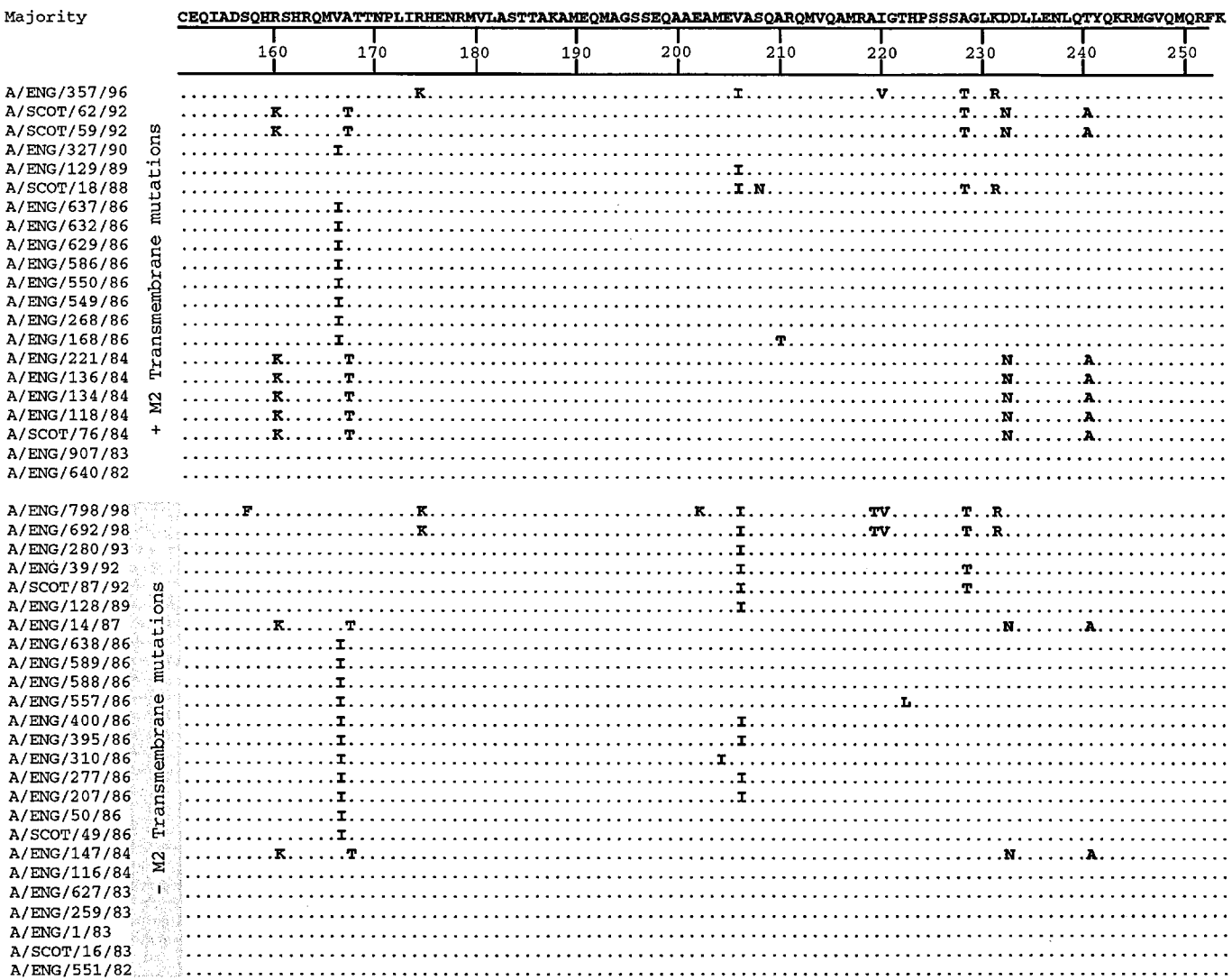
The remaining viruses that had been sequenced did not contain any mutations within the transmembrane domain of M2. This was interesting because the viruses were phenotypically resistant but genotypically they appeared to be sensitive. In order to determine whether there were any changes within M1 that might have influenced the sensitivity of the virus to amantadine, the M1 of all resistant viruses were sequenced. The only virus that could not be used for M1 analysis was A/SCOT/77/92 because the AMPB and AMPCII primers (Chapter 2, page 48) used in the sequencing of the gene failed. It was not possible from the available fragments to piece together the M1 sequence therefore the virus was omitted from the analysis. Alignments were prepared for both nucleotide and amino acids; viruses were grouped into those containing mutations within the M2 transmembrane region (+M2 TM) and those without (–M2 TM). It was hoped that by analysing the alignments for both sets of viruses, anomalies between them would identify possible mutations that might be the alternative cause of resistance other than M2 transmembrane mutations. Alignments of the 759-nucleotide M1 sequence (residues 26-784) were prepared and analysed (Figure 4.14). Initially, it was apparent that within both sets of viruses, the N-terminal third of M1 was highly conserved compared to the C-terminal third of the gene where there was much greater variability between the viruses. When the two groups of viruses were compared, only six amino acid substitutions at five different positions were discovered in the +M2 TM viruses that were absent in the –M2 TM. The majority of these mutations were found at separate amino acid positions in a small proportion of viruses lacking mutations within the transmembrane region of the M2 protein. All other mutations present within M1 could be found in strains of either of the two sets of viruses demonstrating that the mutations were not affecting the susceptibility of the viruses to the drug. Therefore, these results suggested that –M2 TM viruses did not contain mutations within M1 that were responsible for causing resistance to amantadine.

FIGURE 4.14 Amino acid alignment of M1 from amantadine-resistant viruses



Viruses are grouped according to M2 transmembrane mutations; + M2 transmembrane mutations (yellow; top) or – M2 transmembrane mutations (blue; bottom).

FIGURE 4.14 Amino acid alignment of M1 from amantadine-resistant viruses (continued)



Viruses are grouped according to M2 transmembrane mutations; + M2 transmembrane mutations (yellow; top) or – M2 transmembrane mutations (blue; bottom).

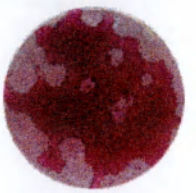
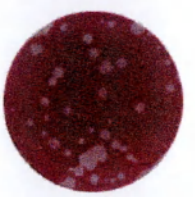
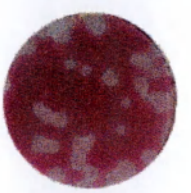
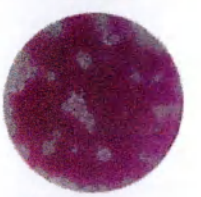
4.3.5 Analysis of Phenotypic and Genotypic Data from Resistant Strains

The phenotypic results obtained from Chapter 3 (Tables 3.7 and 3.8; pages 75 and 80, respectively) were analysed in collaboration with the sequence data of the resistant viruses. Viruses carrying mutations within the transmembrane domain were confirmed to be phenotypically resistant (Table 4.4). The group of viruses that were found to lack mutations within the transmembrane domain of M2 were also phenotypically resistant (Table 4.4; only one virus, A/ENG/588/86, from this group is illustrated). The data collected in Chapter 3 concerning the morphology of plaques was compared to the genotypic results to identify any homology between characteristics.

One interesting observation made was that when viruses carrying a Ser31Asn substitution were grown in the presence of amantadine, the level of inhibition of these viruses was less compared to other resistant genotypes. It was apparent that a relatively large proportion of them (4/9) demonstrated an increase in PFU/ml in the presence of drug although these experiments were not repeated and therefore were treated with some caution. However, based on the available data, it did appear that the presence of the Ser31Asn mutation conferred a selective advantage when grown in the presence of amantadine.

There did not appear to be any other correlations between the resistant genotype and the relative growth properties of the virus. To substantiate these findings and confirm the current results, the plaque reduction assays for each virus would have to be repeated, ideally in the presence of a wider range of concentrations of amantadine. However the results obtained were considered to be reliable enough to postulate on the effects of different mutations on functions of the virus.

TABLE 4.4 M2 transmembrane mutations and associated plaque morphology

Amino acid position and mutation	Virus	Plaque morphology	
		- Drug	+ Drug
No Mutation (sensitive)	A/ENG/290/87		
Val27Ala	A/ENG/357/96		
Val27Ile	A/ENG/586/86		
Ser31Asn	A/ENG/268/86		
Leu36Val Leu43Ile	A/SCOT/77/92		
Leu36Val	A/ENG/134/84		
No Mutation (resistant)	A/ENG/588/86		

Only five viruses with M2 transmembrane mutations from the present study are illustrated. Viruses were grown in MDCK cells in the absence and presence of 1.0 µg/ml amantadine and stained after 72 hours.

4.4 Discussion

The genetic characterisation of the matrix genes from viruses that had been determined as phenotypically resistant to amantadine contained several interesting features. Nucleotide sequencing of M2 revealed the amino acid sequence of each virus tested; this data was also analysed for each functional domain of M2 to correlate findings with functions of the gene.

4.4.1 Extracellular Domain

The extracellular domain of M2 from amantadine-resistant viruses was highly conserved at the amino acid level. This was in agreement with previous studies (175, 412). At the nucleotide level there was a high proportion of synonymous changes (silent) and this has been previously described in other RNA viruses as indicative of proteins that are not under any selective pressure (301). The conserved nature of this domain suggested that its function was critical in the replication cycle of the virus. Within the extracellular domain, two important residues, Cys-17 and -19 were absolutely conserved (411). They have been proposed as the sites where cysteine linkages are formed, holding the structure of the M2 tetramer together (348, 411). Therefore, the results from this study provide further evidence for their importance in the structure and function of the virus.

4.4.2 Transmembrane Domain

Analysis of the transmembrane domain revealed there were higher levels of mutation than in the extracellular domain. There was a higher frequency of non-synonymous changes suggesting an increased selective pressure on this region of the protein. This was expected because it was known that the transmembrane domain of the M2 protein was the target of amantadine and therefore this positive selective pressure would force the virus to mutate, possibly creating a resistant genotype. The ratio of transitions to transversions in the transmembrane region was higher than the other two domains. The significance of this finding was not absolutely clear but it was possible that it provided evidence for the ability of M2 gene to withstand mutations that may have caused structural or conformational

changes to the protein. This may have provided further evidence for the selective pressure on this region; a higher rate of transversion mutations would be indicative of changes occurring that might have provided an advantage to the virus.

Amantadine-resistance is conferred by mutations occurring within the M2 transmembrane domain at one of five amino acid positions (118, 142). Sequencing M2 from the resistant viruses in this study revealed that 22/47 viruses carried mutations within the transmembrane domain with 13/22 carrying a mutation at an amino acid position known to confer resistance. The most common mutation found in H3N2 M2 was Ser31Asn; this correlated with previous studies that had determined that this mutation was frequently isolated in resistant human viruses (90, 144, 147, 166, 417).

Another mutation found during the present study that had been previously reported in resistant human strains was Val27Ala (147, 166, 238). However, at this same residue another mutation was discovered, a Val27Ile substitution that was present in three viruses. The amino acids Ala and Ile are relatively similar in structure; both are non-polar hydrophobic molecules with aliphatic side-chains and a molecular weight of 89 and 131 Daltons respectively (314). Therefore, the similarity between the two molecules in respect to their biological nature suggests that the consequence of substituting the wild-type Val-27 with either Ala or Ile would have a similar effect on the structure/configuration of the ion channel, i.e. resistance to amantadine.

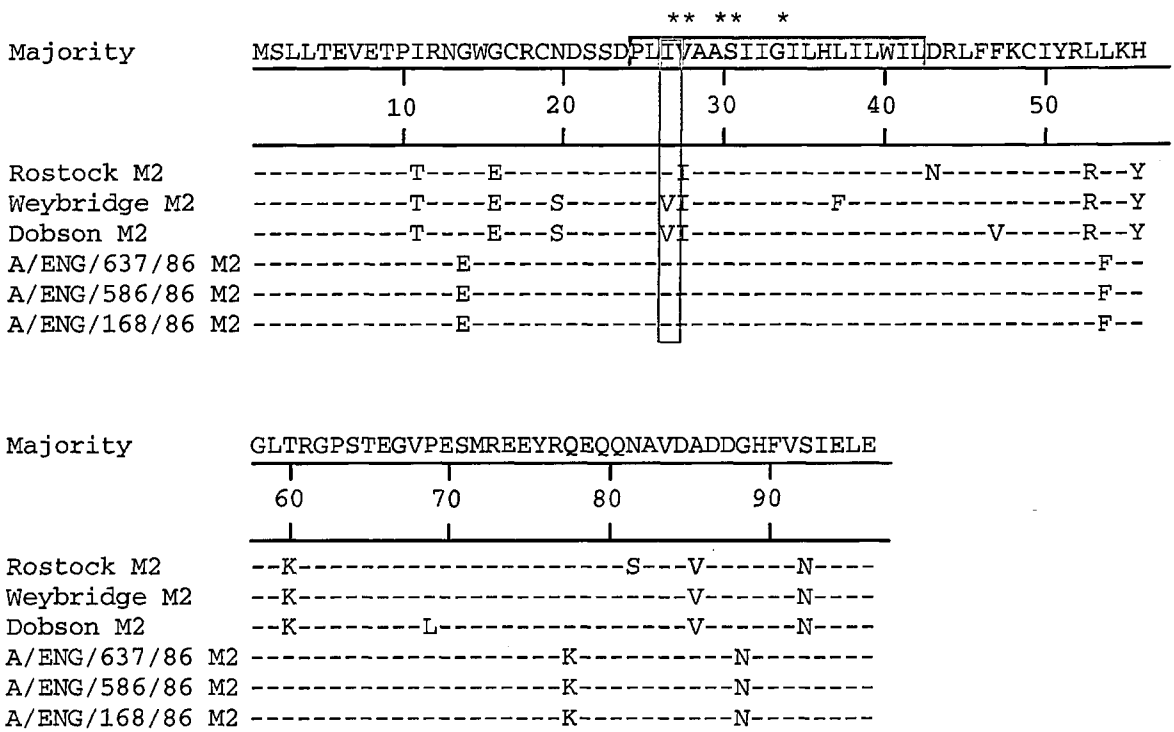
The Val27Ile mutation was interesting because it had not been previously observed in human resistant strains, but is present in some avian strains of influenza A e.g. A/CK/GERM/34 (Rostock) that has a naturally occurring Ile-27 (118, 142). It was known that mutations occurring at position 27 conferred amantadine-resistance in both human and avian strains (25, 118, 147, 166) but it was interesting that the mutation found in the three resistant human viruses (Val27Ile) did not appear to confer resistance in Rostock. The sequence divergence between the matrix genes of avian and human viruses is reportedly in the range of eleven to 22 percent (88). In order to confirm that the M2 of the human strains containing Ile-27 had not been the result of laboratory contamination e.g. through reassortment, an alignment of the whole M2 amino acid sequence of three avian strains and the three human viruses carrying the

Val27Ile substitutions was prepared. This, together with a phylogenetic comparison determined that there were sufficient differences between the two species of M2 to conclude that they were of separate origins (Figure 4.15). It was found that the remaining residues within the transmembrane sequence of M2 were homologous between Rostock and the human viruses, therefore differences between the two strains of virus were put down to other mutations within M2. If any of the other mutations within Rostock M2 had altered the structure or configuration of the channel this might have resulted an amantadine block resulting in a sensitive virus. It would have been interesting to take gene segment seven from the resistant human strains discovered in the present study, and engineer it into a Rostock virus using reverse genetics to investigate whether the progeny virions were of sensitive or resistant phenotype and genotype.

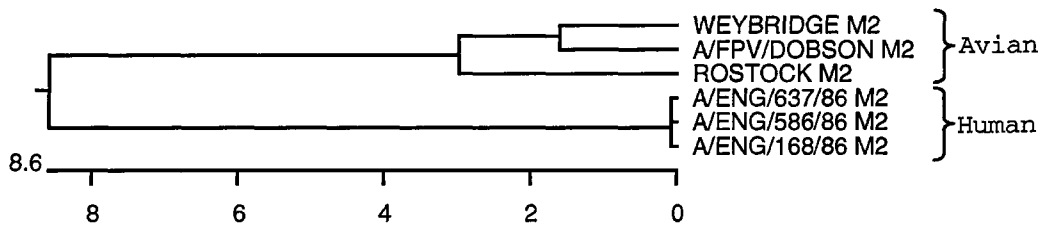
Another aspect that had to be considered was the possible influence of the mutations within the haemagglutinin gene of the Rostock virus. It had been previously demonstrated that mutations within the HA gene affected the susceptibility of Rostock viruses to amantadine (118). Therefore, it was postulated that there could have been interactions between the M2 and HA of Rostock strains which could have maintained the susceptibility of the virus to amantadine.

FIGURE 4.15 (A) Amino acid alignment and (B) phylogenetic analysis of M2 sequences from avian and human influenza viruses

A



B



The transmembrane region (residues 25-43) is highlighted and individual residues known to be involved in conferring amantadine-resistance are marked with an asterisk. The amino acid residue at position 27 is boxed in red.

The mutation Leu36Val was found in 8/22 H1N1 resistant viruses with mutations in the transmembrane domain of M2. There is no previous evidence to suggest that this mutation had occurred in either human or animal influenza A strains and therefore it was considered to be novel. It was interesting to note that the Leu36Val mutation was situated in close proximity to the His-37 molecule located within the transmembrane pore. His-37 has been implicated as having an essential role in the gating of the M2 ion channel (281). Therefore, it was possible that a mutation occurring immediately next to the His-37 residue might have indirectly affected the function of the channel by interfering with this residue. It was thought that this mutation might have been the cause of the amantadine-resistance observed in the viruses that carried it, but upon sequencing a selection of H3N2 and H1N1 sensitive viruses it was apparent that the mutation was found in both sensitive and resistant H1N1 strains (Figure 4.13). This finding was interesting because it disproved the initial hypothesis. It appeared that the presence of the mutation was not the determining factor in these viruses that was controlling their susceptibility to amantadine. The substitution of the Leu residue for a Val residue at position 36 was examined. The structures of the two amino acids were similar; both are hydrophobic molecules with aliphatic side chains, each with a molecular weight of 131 and 117 Daltons respectively (314). This suggested that the structural differences between the two substituted amino acids at this position were unlikely to cause a significant conformational change within the channel at that position which might have affected the gating function of His-37.

In order to further explore this finding, a series of experiments would need to have been performed. Initially, inserting gene segment seven carrying the Leu36Val mutation into a known amantadine-sensitive parent strain, and screening the progeny for resistance would have confirmed whether the mutation was responsible for conferring resistance. In order to ascertain whether the mutation had any affect on the configuration of His-37 and therefore the function of the channel, viruses with the Leu36Val mutation could have been used in experiments similar to those previously described (253, 281) and the currents across the channel measured. Results from these proposed experiments would have revealed whether the close proximity of the Leu36Val mutation to the His-37 residue affected the function of the ion channel.

An observation made during the analysis of the M2 amino acid alignments revealed that between all of the resistant viruses, the His-37 residue was absolutely conserved. The significance of this finding was important when related to previous studies that have investigated the function of His-37 within the regulation of protons across the channel (281, 321, 375). When the sensitive viruses were sequenced, it was also found that His-37 was absolutely conserved. These data were further evidence of the importance of His-37 in controlling the maintenance of pH during specific areas of the virus replication cycle. Mutant ion channels with His-37 substituted for other amino acids do not become protonated and are not activated by low pH (375).

There were a relatively large number of viruses selected from this study that were amantadine-resistant by phenotypic assays but when characterised genotypically, they lacked any mutations within the M2 transmembrane domain. The reasons for this phenomenon are unclear but several theories may be postulated. From the phenotypic testing of the resistant strains in Chapter 3, it was apparent that some viruses were composed of mixed populations of genotypes (Table 3.8, page 80). It seemed possible that upon PCR amplification of the mixed population, a sensitive virus was the template amplified, which would have resulted in the amplified product consisting of an M2 protein lacking the mutations known to confer resistance. A way of avoiding this situation would have been to titrate the virus in the presence of amantadine and pick individual plaques from the progeny of viruses produced. This process would have ensured that any genetic material used would have originated from a virus that had grown in the presence of drug. Performing PCR amplification on the plaque-purified material would have made certain that the amplicons that were produced originated from a resistant virus.

Another possible source of error was from the growth of the resistant viruses in tissue culture. After the initial testing of the viruses by EIA and plaque reduction assay, it was necessary to grow each strain in tissue culture to produce enough material for genetic characterisation. Therefore, between the phenotypic and genotypic testing the viruses were subjected to a passage without the selective pressure of drug. This may have resulted in a shift in the proportion of sensitive and resistant genotypes within a given mixed population of one virus strain. Ideally, to characterise the exact genotype of the virus it would have been necessary to test original material obtained from the patient. To enable the

detection of the virus within the laboratory, the process of amplification in tissue culture was necessary and this process may have altered the balance between resistant and sensitive genotypes within each virus culture. Unfortunately, for the majority of viruses screened in this study, the original isolates were not available. Previous work has shown that resistant strains can either maintain resistance or revert to the sensitive wild-type when the selective pressure of amantadine is removed (22, 118). An option would have been to grow the resistant viruses in medium containing drug but it was thought that this might have altered the natural resistant genotype of the viruses. Each virus upon amplification in tissue culture, prior to genetic characterisation should have been tested phenotypically to confirm that the resistant phenotype had been maintained during the passage. However, this would have been extremely labour intensive and therefore was not practical to perform.

Other proteins closely linked to M2 during the replication cycle of influenza A include M1 and HA. It was thought that changes within these proteins might have had an indirect effect upon the susceptibility of the viruses to amantadine. During the initial stages of replication, the M1/RNP complex dissociates releasing the RNP into the host cell cytoplasm and subsequently the host cell nucleus (136). The dissociation of the complex is triggered when the endosomal pH is reduced; reduction in pH is caused by the influx of protons into the endosomal interior through the M2 ion channel (414). When amantadine blocks the M2 ion channel the pH remains relatively stable and therefore the M1/RNP complex cannot dissociate. It was postulated that changes within the M1 protein might raise the pH threshold at which the dissociation event occurred, thereby allowing the release of RNP without the requirement of low pH. However, sequencing the M1 from all resistant strains revealed that there were no sequence differences between + M2 TM viruses and – M2 TM viruses, suggesting that – M2 TM viruses did not contain mutations within M1 that were responsible for causing resistance to amantadine (Figure 4.14). Therefore, to further test this hypothesis concerning the dissociation of the M1/RNP complex, other aspects of the event would need to be investigated.

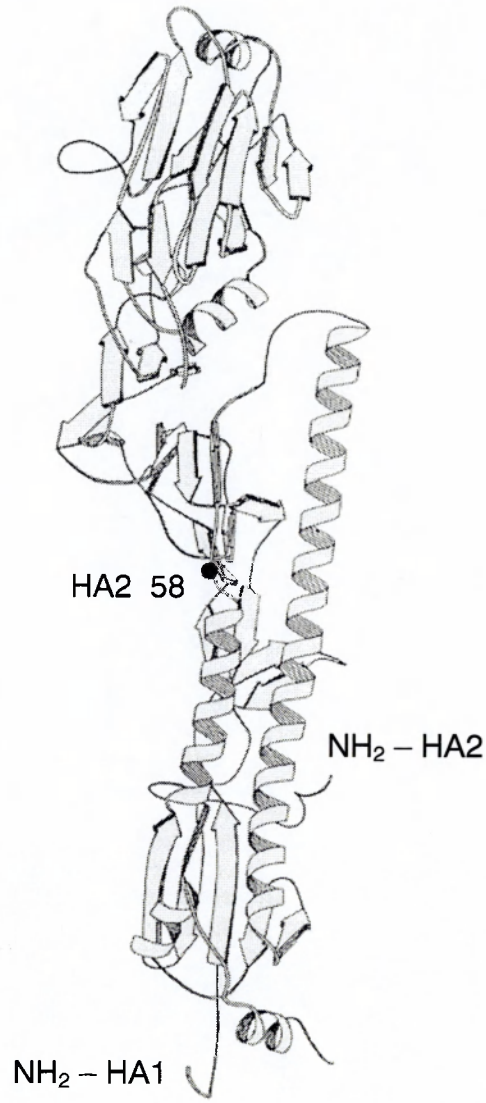
Another possibility for the absence of M2 transmembrane mutations in phenotypically resistant viruses involved the close association of the activities of HA and M2 during the influenza A replication cycle. Previous findings had reported that mutations within the HA2 region of the HA gene complemented mutations

within the M2 transmembrane domain (118). The mutations within HA2 were found to affect the fusion properties of the protein to the extent that the HA precurser could undergo normal fusion events, even when the M2 ion channel was blocked by amantadine therefore lowering the pH of some of the compartments of the TGN involved with HA maturation (118).

In a study of amantadine-resistant strains of the avian influenza virus Rostock, Steinhauer *et al.* (1991) found that when the resistant viruses were characterised, three viruses were genetically distinct due to an absence of any mutations within the M2 transmembrane domain (340). The HA of these viruses were analysed and it was found that they all shared a common mutation within the HA2 region. This mutation (Lys58Ile) was found to generate a HA species that was more acid-stable resulting in a protein with a lower fusion potential than the wild-type (Figure 4.16). This finding suggested that in these viruses, the fusion potential of the HA was lower to compensate for the blockage of the M2 ion channel caused by amantadine. This phenomenon had not previously been reported in human viruses. However, it may be that several of the viruses from this study, which had no mutations within the M2 transmembrane region but were resistant by phenotypic methods, might have contained mutations within the HA to compensate for increased acidity in the TGN. A drawback with this theory was that the previous work was carried out on avian strains of influenza; these strains had HA species that were cleaved proteolytically within the TGN by the protease furin. The avian HA was therefore susceptible to acidic conditions and upon exposure to lowered pH within the TGN, it was converted to a low pH form which prevented the release of progeny particles from the infected cell (310, 346). The primary effect of amantadine on human influenza A viruses is preventing the dissociation of the M1/RNP complex during the initial stages of the replication cycle (143, 184). The HA of human influenza A viruses do not contain the multibasic cleavage site that is present within certain avian strains (182). The precursor of HA (HA0) does not undergo the proteolytic cleavage into the HA1 and HA2 subunits intracellularly, therefore if the pH of the TGN was lowered this should not affect the formation of native pH. Therefore, theoretically, if mutations existed within the human HA2, similar to those found in Rostock HA2 it would not have the same compensating effect on the human viruses. To investigate this phenomenon further, it was proposed that the HA, in particular the HA2 domain, of the resistant viruses lacking M2 transmembrane mutations would be sequenced to identify any common

mutations at positions that were known to be important in the fusion activity of the protein. Comparisons of these sequences with the HA of known amantadine-sensitive viruses should also help to identify mutations that might have occurred.

FIGURE 4.16 Structure of the influenza A HA monomer



The figure represents the structure of the HA monomer, showing the location of the mutation found at residue 58 of HA2 that can indirectly confer amantadine-resistance. Also shown are the amino terminals of HA2 and HA1. Taken from (340).

4.4.3 Cytoplasmic Domain

Analysis of the sequence data for the M2 cytoplasmic domain demonstrated that it was relatively conserved in relation to the size of the domain, but not to the same degree as the extracellular domain (Figure 4.10 and 4.11). The relatively high proportion of non-synonymous changes observed suggested that in common with the transmembrane domain, this region was subject to some form of selective pressure that was driving the amino acid changes. The transition to transversion ratio was much lower than the transmembrane domain. The cytoplasmic domain was previously shown to contain several important sites for post-translational modifications to occur. The addition of long chain fatty acids had been reported to occur at Cys-50 (347). Results from this study illustrated that Cys-50 was absolutely conserved in all resistant and sensitive viruses demonstrating the importance of this residue in the function of M2.

Within the cytoplasmic domain of M2, three Ser residues, Ser-64, -89 and -93 have been identified as sites where phosphorylation can occur (162). Interestingly, only 1/3 of these Ser residues (position 64) was absolutely conserved between the resistant and sensitive viruses from the present study. The other sites, Ser-89 and -93 were not conserved; 14/47 resistant and 12/37 sensitive viruses (Ser-89), and 1/37 sensitive virus (Ser-93) carried mutations at these positions (Figures 4.10 to 4.13). The Ser-89 residue had been substituted by a Gly (10/14) and an Asn (4/14) in H3N2 and H1N1 resistant strains. Sensitive strains lacking the phosphorylation sites had Ser-89 substituted by Gly (11/12) and Asn (1/12), and the Ser-93 residue substituted by Asn (1/1). These data indicate that although the three Ser residues at positions 64, 89 and 93 had all been implicated as sites for post-translational phosphorylation, it appeared that only Ser-64 was essential for this role. Viruses were isolated lacking Ser at residues 89 and 93 indicating that it was not an absolute requirement for the proteins to be phosphorylated at these sites. However, Castrucci *et al.* (1995) demonstrated that virus mutants containing truncated M2 proteins lacking five and ten residues at the C-terminal end could not be rescued using a reverse genetics system (45). It is possible that the five-residue deletion mutant illustrated the importance of Ser-93, which might explain why only a single virus was found with a mutation at this Ser residue in the present study.

When the viruses lacking phosphorylation sites were further analysed, it was apparent that a high proportion of both resistant and sensitive viruses contained mutations within the transmembrane domain. In total, 27/84 resistant and sensitive viruses lacked a phosphorylation site at either Ser-89 or Ser-93. Of these viruses, 19 had mutations within the transmembrane domain of M2. This was an interesting finding because it appeared as if mutations occurring in the transmembrane region were being complemented by substitutions of Ser residues at either position 89 or 93 in the cytoplasmic tail. In another study of M2 cytoplasmic tail truncation mutants, Tobler *et al.* (1999) demonstrated that truncated M2 proteins had reduced ion channel activity (359). For most truncated mutants it was shown that the observations were a possible consequence of reduced protein expression at the infected cell surface, thereby reducing the total capacity for conductance to occur. However, one mutant demonstrated good expression while maintaining reduced ion channel activity (359). It was concluded that the truncation of the M2 cytoplasmic tail at position 82 had caused an alteration in the structure of the ion channel formed by this mutant, which consequently reduced the potential for ion flow through the channel. It is interesting that this mutant did not contain either the Ser-89 or -92 residues. Therefore, it is possible that the loss of one or more phosphorylation sites within the C-terminal third of the virus has an effect on the ion channel activity of the protein. In the present study, the mutations found in the transmembrane region of viruses containing a Ser-89 substitution might have been compensating for structural changes in the ion channel caused by the Ser substitutions. Therefore, the M2 transmembrane mutations were possibly acting to stabilise the channel and maintain normal activity and the function of the protein. This is an interesting idea that could be further investigated using site-directed mutagenesis and ion channel conductance studies to assess the possible relationship between Ser-89 and -93 substitutions and ion channel activity.

Evidence for the previous occurrence of human influenza A viruses lacking the Ser-89 or Ser-93 residues was found. The two ancestral H1N1 reference strains, A/WSN/33 and A/PR/8/34 were both found to contain a Gly residue at position 89 within the M2 cytoplasmic tail (233, 397). This was interesting because it demonstrated that human viruses lacking this phosphorylation site had circulated previously. The resistant viruses found in the present study carrying a substitution at Ser-89 were predominantly H1N1 subtypes but there were two viruses of H3N2 subtype demonstrating that this trait was not exclusive to H1N1 viruses. Another

interesting observation was that A/WSN/33 and A/PR/8/34 both had mutations within the transmembrane domain of M2 that caused amantadine-resistance in both strains; how these viruses acquired resistance was unclear because their parental origins were isolated before the use of amantadine or rimantadine. Therefore, these two early H1N1 strains were amantadine-resistant and lacked the Ser-89 phosphorylation site, a feature in common with 14 resistant viruses isolated from the present study. It is possible that there may be a connection between the two processes of phosphorylation and generation of amantadine-resistance through mutations in the transmembrane region. It is possible that the substitution of the Ser-89 with other amino acid residues, which resulted in an overall reduction in phosphorylation of the protein, in some way altered a functional or structural aspect of the ion channel, the domain primarily responsible for conferring amantadine-resistance.

4.4.4 Genotypic and Phenotypic Correlation

The findings from the analysis of both genotype and phenotype of resistant viruses produced an interesting result. Although the data needed to be repeated to provide more substantial evidence, it was possible that the viruses with a Ser31Asn substitution replicated at higher rates in the presence of amantadine. If this finding could be confirmed it would support previous evidence for the prevalence of the Ser31Asn mutation in resistant human influenza A viruses (90, 144, 147, 166, 417). It suggested that viruses of this resistance genotype had a selective advantage over others and therefore could out-compete other genotypes where conditions were favourable. However, the data presented here represents the growth of the viruses *in vitro*, further work would be required to confirm this *in vivo*. A possible way of investigating this might be to infect ferrets with viruses containing wild-type M2, and M2 with a Ser31Asn mutation. The viruses could be constructed by simple reverse genetics techniques to ensure that no other variable influenced the results i.e. viruses would have the same backbone, but contain sensitive and resistant mutations within M2. This type of experiment should provide data on the growth potential of viruses carrying the Ser31Asn mutation compared to wild type sensitive strains *in vivo*.

It is difficult to postulate why viruses with the Ser31Asn mutation had an apparent evolutionary advantage over other resistant viruses. It is possible that the mutation occurring at that specific position within the M2 ion channel brought an added stability or rigidity to the channel, making the whole protein structure more stable and therefore less likely to fail in its function to control the pH of certain cellular compartments. Viruses that became resistant through mutations at other positions within the ion channel might have contained inherently unstable channels that would have reduced the ability of the virus to replicate and therefore compete with other genotypes. It is certain from previously published evidence that viruses with the Ser31Asn mutation have an increased ability to survive in, and transmit between humans but the underlying mechanisms remain unclear (90, 144, 147, 166, 417).

4.4.5 Concluding Remarks

In summary, the genotypic characterisation of the resistant viruses selected from EIA and plaque reduction assay revealed mutations within the transmembrane domain of M2 that could be implicated in drug-resistance, or were present in both sensitive and resistant viruses. The group of viruses lacking mutations within the domain suggested that interactions with other viral gene products might influence the susceptibility of the virus to the drug. Sequence data confirmed that the extracellular domain was highly conserved in all viruses used in the study but the transmembrane and cytoplasmic domains showed differing degrees of variation. Important residues previously reported to be involved in post-translational modification of M2 were conserved demonstrating that certain regions of the M2 protein are essential for replication of the virus.

Chapter 5

Evolution of Matrix Genes from Influenza A Viruses isolated in the United Kingdom 1968-1999

5.1 Introduction

As described earlier (section 1.6.3) RNA viruses are unique with respect to the high rates of mutation occurring during replication. The RNA polymerase molecule present in these viruses has low fidelity (it lacks 3'→ 5' exonuclease activity) that results in the occurrence of a high error rate during the process of genomic transcription (338). The rate of mutation in RNA viruses can range from 1×10^{-3} to 1×10^{-5} misincorporations per base site per round of copying (78) compared to the low mutation frequency associated with DNA replication, which can be as low as 1×10^{-8} to 1×10^{-11} (159). The high error rate and relatively high levels of replication demonstrated by these viruses can lead to the formation of a population of viruses containing diverse variants which are all phenotypically related but carry distinct genomic sequences as a result of mutation. The phenomenon of diverse RNA populations has been referred to as "quasispecies" (83).

5.1.1 Evolution of Influenza A Genes

Influenza A viruses are able to undergo rapid evolution due to the high error rate during the replicative processes of the virus. This property allows the virus to continually change its genetic makeup to evade such selective pressures as the immune response and antiviral drug treatment. The rate of change for different influenza A proteins is dependent on the function and the location of the protein within the virion structure (Table 5.1).

TABLE 5.1 Evolutionary rates of change for influenza A genes

Gene Segment (Subtype)	Host	Rate of Change (site/year x 10^{-3})		N	Period	Reference
		Nt	aa			
HA (HA)*	Human	3.65	5.83	183	1968-1994	(59)
HA (H3)*	Human	7.00	-	14	1968-1980	(34)
HA (H3)*	Human	5.7	9.7	254	1984-1996	(96)
HA (H3)	Equine	2.80	-	2	1963-1979	(63)
HA (H1)*	Human	4.30	-	14	1977-1983	(294)
NA (N2)	Human	2.28	2.56	38	1968-1995	(401)
NA (N2)	Human	3.7	4.7	6	1957-1979	(313)
NS	Human	1.94	-	9	1942-1985	(41)
NS	Human	1.83	-	15	1933-1977	(180)
NP	Human	2.18	0.42	16	1933-1983	(111)
NP	Swine	2.12	0.22	11	1930-1988	(111)
NP	Avian	1.21	0.00	19	1927-1982	(111)
PA	Human	1.32	-	4	1934-1968	(264)
PB1	Human	0.87	-	3	1957-1968	(181)
PB2	Human	1.82	-	7	1933-1988	(114)
M	Human	1.08	-	13	1933-1988	(175)
M1	Human	0.83	0.03	13	1933-1988	(175)
M2	Human	1.36	0.46	13	1933-1988	(175)
M	Swine	1.30	-	8	1930-1988	(175)
M1	Swine	1.43	0.05	8	1930-1988	(175)
M2	Swine	0.91	0.48	8	1930-1988	(175)

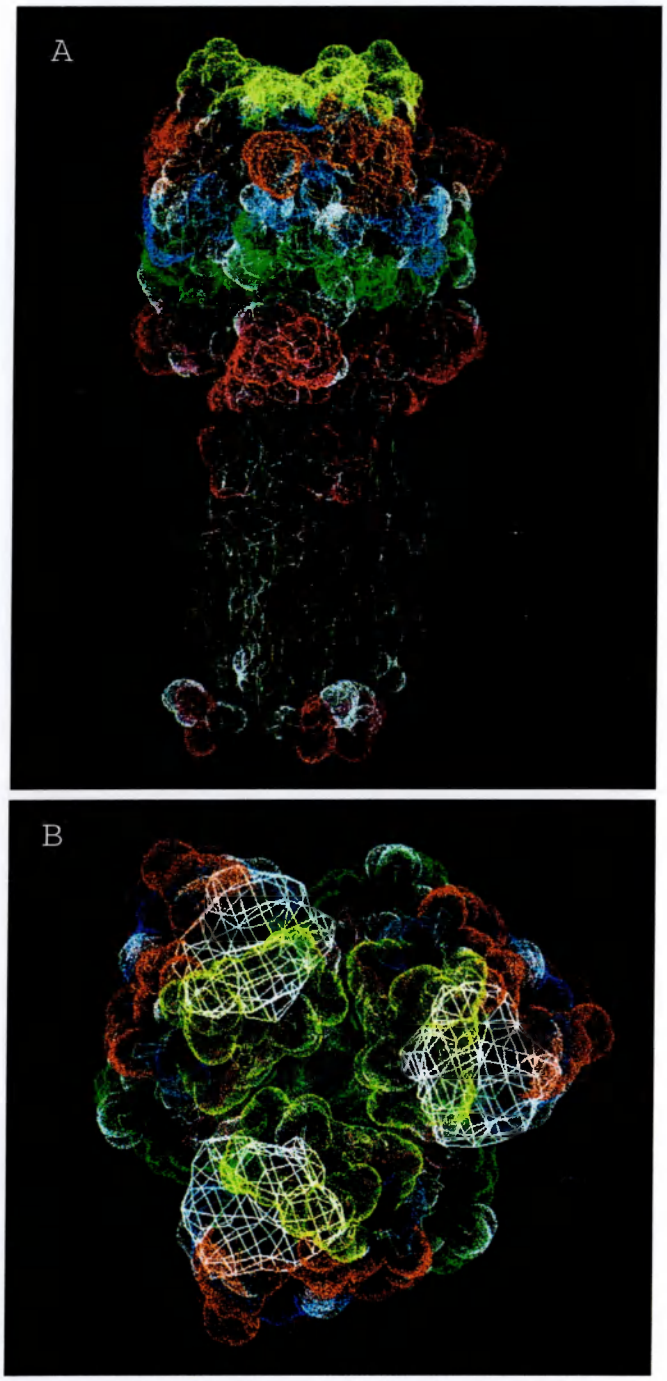
*** Based on the rate of change within the HA1 region of the HA molecule. Adapted from (113, 383). N – number of viruses analysed.**

5.1.1.1 Haemagglutinin (HA)

The protein reported to be undergoing the most rapid rate of change is the HA molecule, located at the surface of the influenza A virus envelope. There has been some controversy associated with the evolution of this molecule; several theories have been postulated as to how the HA of human viruses can change so rapidly. One such hypothesis supports the neutral theory of evolution where it is believed that the mutation rate of HA is simply higher than other genes: consequently, the rate of neutral changes is high, with a proportionally high frequency of coding changes (110). This theory is supported by evidence that shows within all influenza A genes the rate of synonymous changes is always higher than non-synonymous (110).

Alternatively, it could be that the HA molecule has a higher rate of mutation, but in addition as there is positive selection for coding changes that are beneficial to the virus and confer a selective advantage (345). Evidence to support this hypothesis can be found when the distribution and frequency of silent and coding changes are analysed within the HA1 subunit. The rate of non-synonymous changes is much higher, and synonymous changes lower within antigenic regions of HA1 (173). Therefore, this suggests that positive pressure has forced the selection of viruses containing coding changes within the HA1 antigenic regions, most probably because these changes confer a selective advantage to the virus (Figure 5.1).

FIGURE 5.1 Antigenic variation in influenza A H3 strains from 1968-1995



Residues that have changed over this time are represented by a dot surface. Antigenic sites located on HA1 are represented by designated colours; A, red; B, yellow; C, magenta; D, cyan; E green; and positions not assigned to an antigenic site in white. (A) The HA polypeptide is illustrated and (B) the distal globular head domain of the H3 HA with the receptor binding pocket represented by the white mesh grid. Adapted from (59).

The overall rate of change of HA is higher compared to the other external and internal influenza A genes (Table 5.1). An estimate of the rate of change of the HA1 subunit of the HA molecule ranges from 4.3 to 7.0 x 10^{-3} changes per nucleotide site per year with the average value around 5.7 x 10^{-3} changes per nucleotide site per year (34, 96, 294). The HA has been identified as the main determinant of immunity to influenza in man; it is thought to stimulate the initial development of neutralising antibody, which provides the first stage in the immunological response to infection (286). Therefore, the selective pressure applied upon the HA, especially within the main antigenic sites located within the HA1 subunit, is sufficient to force the selection of antigenic variants. This gradual accumulation of mutations within the HA causes subtle variations within the antigenicity of the virus, enough to enable the virus to evade the immune response; this process is referred to as antigenic drift (59).

5.1.1.2 Neuraminidase (NA)

The NA molecule represents the second major glycoprotein located on the surface of the influenza A lipid envelope. In comparison to the HA molecule there have been relatively few studies to determine the evolution of this gene. Early work had demonstrated that drift could be observed in the NA and it appeared to be as a result of similar processes affecting the HA (209). Although anti-NA antibodies reportedly do not neutralize virus activity, it is thought that they do play an important role in the evolution of NA (189).

Studies of the evolution of human NA have extensively focused on the N2 subtype (209, 237, 401). The rate of evolution of N2 is reported to be in the range of 2.28 to 3.70 x 10^{-3} changes per site per year for nucleotides and 2.56 to 4.7 x 10^{-3} changes per site per year for amino acids (313, 401). It has also been reported that the NA gene of human H3N2 viruses has evolved in two separate lineages (401). Work to determine species-specific lineages for NA has been performed by Saito *et al.* (1993) who studied the N8 influenza viruses (313). It was found that N8 had evolved into three distinct lineages; equine, Eurasian avian and North American avian. The rate of evolution for N8 in equine viruses was estimated to be 2.06 x 10^{-3} and 1.75 x 10^{-3} changes per site per year for nucleotides and amino

acids, respectively (313). When compared to the rate of human N2 evolution, equine N8 was lower suggesting that within humans the selective pressure exerted on the NA was higher than in equine viruses. Studies of the N8 found in avian lineages demonstrated that, as with studies of other influenza A genes, there was a high degree of conservation of amino acids within the protein suggesting that the NA in avian strains was at an evolutionary optimum and that changes were detrimental to the survival of the virus. It was also proposed that that the reported differences between human and equine NA were possibly attributed to the length of immune response following vaccination in both respective species. The effect of the vaccine in horses is short-lived, as little as three months compared to the human response, which lasts significantly longer (313). Therefore it was proposed that the increased length of immune pressure exerted on the virus in humans might have produced the observed results i.e. higher rate of NA evolution in humans compared to horses (313).

5.1.1.3 Non-structural (NS)

There have been several studies involved with predicting the evolution of the NS gene of influenza A. Similar to gene segment seven, the NS gene (RNA segment 8) encodes two overlapping proteins, NS1 and NS2 (201). NS1 has been shown to interact with host nuclear proteins and evidence suggests that NS2 interacts with the M1 protein (288, 403).

Phylogenetic analysis has shown that influenza A NS is categorised into species-specific lineages: human, equine and distinct avian lineages (180, 226). Within human influenza A viruses, the NS gene evolved as a single lineage until 1977 at which point H1N1 viruses re-emerged. Following this event, human NS genes have evolved independently in the H3 and H1 subtypes (226). The evolutionary rates of NS and its gene products follow similar species-specific rates of change when compared to other influenza A internal proteins e.g. the NP gene. In terms of the nucleotide rate of change, the fastest is observed in the human lineage and slowest in the equine lineage (180). The rate of change of amino acids for NS was lowest in the avian lineages compared to human and equine (180).

It has been shown that analogous to the M1 and M2 proteins, the two gene products of NS evolve at different rates. Interestingly, it appears that the rates at which NS1 and NS2 evolve are dependent on species lineage (180). It has been proposed that differences observed may be attributed to the functions of both proteins. NS1 interacts with host nuclear proteins and therefore in different species undergoes different changes in order to achieve optimal interaction (180). Estimates of the rate at which the human NS gene is evolving range from 1.83 to 1.94 x 10^{-3} changes per nucleotide per year (41, 180). This rate is comparable to other internal genes of influenza A, but lower than the surface glycoproteins (Table 5.1).

5.1.1.4 Nucleoprotein (NP)

The NP gene of influenza A viruses has been previously shown to be a major determinant of host specificity (318). The important role of this gene has led to several studies that have investigated its evolution. It is interesting to note that the NP gene has undergone a relatively constant rate of change that has remained, even during the antigenic shift events of 1957 and 1968 (328). The rate of change of the NP gene is estimated to be in the range of 1.62 to 2.2 x 10^{-3} changes per nucleotide site per year for the human lineages, 1.21 to 1.39 x 10^{-3} changes per nucleotide site per year for avian and 0.78 x 10^{-3} changes per nucleotide site per year for the equine lineage (5, 111, 112). Analysis of amino acid changes demonstrates that evolution within the avian lineages is virtually static with practically no changes occurring over a period of approximately 50 years (-0.02 x 10^{-3} changes per amino acid site per year) with human and equine viruses undergoing slightly higher rates of 1.14 x 10^{-3} and 0.66 x 10^{-3} changes per amino acid site per year respectively (112).

The NP gene has been shown to exist in different lineages at both the nucleotide and protein level (112). Similar to other influenza A proteins, host specific lineages appear to evolve at different rates. Nucleotide sequences of NP from human and swine viruses have demonstrated that they share a common ancestor. It has also been shown that these two lineages have evolved at a higher rate than the avian NP gene (111). Therefore, as with other influenza genes, it has been hypothesised

that within avian lineages, the NP gene is maintained at an adaptive peak because this lineage represents the ancestral origin of the gene. However, rates are higher within human and equine lineages because the virus has had to adapt its host specificity and immune response.

5.1.1.5 Polymerase Complex (PA, PB1, PB2)

The three subunits comprising the polymerase complex in influenza A viruses have each been studied to estimate the origin and evolution of these genes. It has been reported that the PA gene of human influenza A is evolving at a rate of 1.32 x 10^{-3} changes per nucleotide site per year (264, 383). Findings indicated that PA has evolved into at least five different lineages. Human, equine and swine lineages have evolved independently and avian viruses have evolved into two separate lineages. The gene is relatively conserved in humans and appears to have remained in a single lineage despite two major antigenic shifts (1957 and 1968) (264).

The PB1 gene has evolved along four distinct lineages comprising human, avian, equine and swine (181). Interestingly, the single human lineage is comprised only of the H1N1 subtype of viruses; H3N2 and H2N2 viruses were found to be more closely related to the avian lineage providing evidence that prior to the 1957 antigenic shift, avian PB1, together with HA and NA was introduced into humans to create the novel pandemic strain (181). The evolution of human PB1 has been estimated to be 0.87 x 10^{-3} changes per nucleotide site per year, much lower than that of the human PA gene (181, 383).

The final component of the polymerase complex, PB2, is evolving at an approximate rate of 1.82 x 10^{-3} changes per nucleotide site per year in human influenza A viruses (114). This figure is similar to that of the evolution of the PA gene, but is much higher than the PB1 gene. The evolutionary patterns of PB2 genes differ from those found for the PA and PB1 genes. PB2 has evolved into four main lineages; human, two distinct avian and equine lineages. The lineage containing the human strains also contains swine viruses that have a PB2 gene closely related to human strains (114).

In general, the four units comprising the RNP complex (NP, PA, PB1 and PB2) have been found to follow different evolutionary pathways suggesting that selective pressures driving evolution affects the individual subunits and not the complex as a whole (114).

5.1.1.6 M1and M2

There have been relatively few studies which have investigated the evolution of gene segment seven of influenza A viruses. Possibly the most important data were generated by Ito *et al.* (1991) who compared the evolution of the M1 and M2 proteins from viruses of different lineages (175). It was found that the rates of evolution between the two proteins differed, with M1 changing at a much slower rate than M2 (Table 5.1). The rate of change of nucleotides for the human M1 and M2 genes were 0.83×10^{-3} and 1.36×10^{-3} per site per year respectively and 0.08×10^{-3} and 1.38×10^{-3} for amino acids respectively (175).

A more recent study by Park *et al.* (1997) investigated the evolution of M1 that originated from human influenza A viruses (273). The findings from this study indicated that nucleotide changes were occurring at a rate comparable to other studies but there were virtually no amino acid changes recorded over a period of approximately 60 years. From these results it was concluded that the human M1 had actually reached an evolutionary stasis and that any changes to the protein structure would be detrimental to the virus (273).

Analysis of the evolution of the M1 and M2 genes into different lineages show that in respect to amino acid differences, M1 is relatively conserved across human, avian and swine-specific lineages (175). However, M2 proteins have shown a much greater rate of change in lineages containing human and swine viruses but virtually none within avian viruses (175). The M1 and M2 proteins from equine viruses have also been shown to evolve at a much slower rate than human viruses (220). The overall reasons behind these observed differences in evolution in different lineages have been related to lower mutation rates of the polymerase complex during RNA replication and the origin of the influenza A viruses. The low

rate of evolution in avian influenza strains confirms the accepted theory that influenza A viruses originated from an avian lineage. Therefore, it might be concluded that in their original hosts, the virus does not have to evolve to adapt as rapidly as viruses that have jumped the species barrier more recently.

In conclusion, it is apparent that each of the influenza A gene segments evolves at a different rate and in many cases follow different evolutionary lineages. Interestingly, a simple comparison of the silent changes observed within each gene reveals to some extent the degree of evolutionary pressure that a particular gene is under. For example, the human NP and M1 genes have 81 and 96 per cent silent changes respectively over a fifty year period (1933-83) indicating that the amino acid sequences are conserved and not undergoing any significant evolutionary change (383). It may be that these observed evolutionary constraints are due to vital functions associated with the proteins, or that a state of evolutionary stasis has been reached in that species for this gene. The latter point can be illustrated when the evolutionary rates of change for the majority of the influenza A genes are compared between human and avian influenza viruses. In all cases, the rate of change for influenza A genes in respect to amino acid changes per site per year in avian strains is significantly lower than in human strains; genes from avian viruses have often been reported to be undergoing no significant evolutionary change at all. These findings suggest that within these viruses, the viral genes are at an evolutionary optimum and are not forced to undergo change. This is likely to be because the avian reservoir is thought to be the ancestral origin of influenza A viruses, therefore all genes within these viruses are likely to be optimally adapted to the host.

5.2 Aims

There are relatively little data describing the evolution of gene segment seven of influenza A viruses within the human host; there are no reported studies of the evolution of these genes specifically within a defined, geographically-contained population. Utilising the set of sequencing data generated from the work described in Chapters 3 and 4, including sequences of both amantadine-resistant and sensitive viruses, the evolution of gene segment seven over the past 30 years within the UK over a period of circulation of H3N2 and H1N1 viruses was estimated. The main aims of this work were:

- To compare the evolution of M1 and M2; more specifically the evolution of these genes between amantadine-resistant and -sensitive strains and different subtypes to determine whether the phenotype or genotype affect the rate of change within the respective proteins.

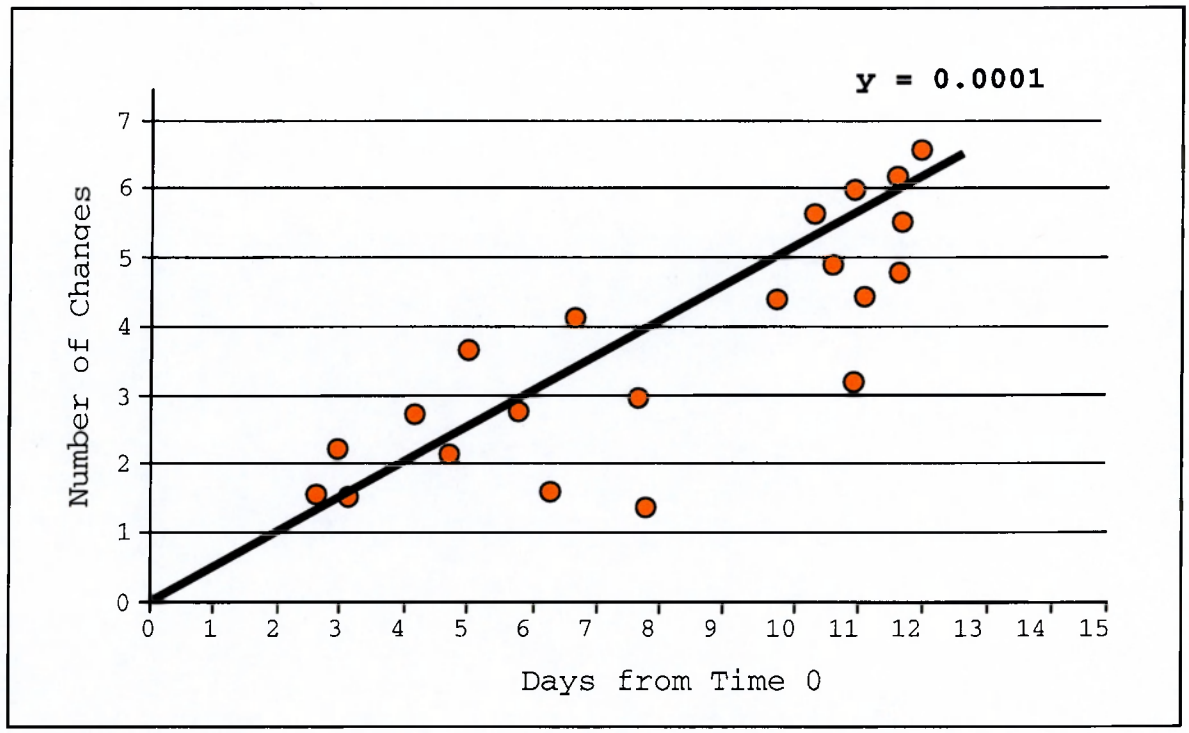
- To determine what lineages viruses have evolved into over the previous 30 years in the UK using phylogenetic analysis. Also, to determine the positions of amantadine-resistant and -sensitive viruses within phylogenetic trees and relate these positions to drug genotype or phenotype.

5.3 Results

5.3.1 Rate of Change of M1 and M2

In order to calculate the rate of change for the M1 and M2 genes of the influenza A viruses sampled during the study, nucleotide alignments for 84 influenza A strains were prepared. Sequences were aligned to an ancestral strain, A/Aichi/2/68 and A/USSR/90/77 for H3N2 and H1N1 viruses respectively. The ancestral strains were chosen because they both represented original prototype H3N2 and H1N1 viruses. A/Aichi/2/68 represented one of the first characterised viruses that appeared when H3N2 viruses superseded the H2N2 subtype in 1968. Although H1N1 viruses circulated the globe from approximately 1918 to 1957, the majority of the H1N1 viruses used in this study were isolated post 1977, when the H1N1 subtype reappeared to cocirculate with H3N2 viruses. Therefore, it was thought that A/USSR/90/77 represented the prototype strain for H1N1 viruses that have been in circulation since 1977. Files containing both H3N2 and H1N1 sequences were aligned to the majority sequence calculated by the clustal algorithm in MegAlign. Synonymous and non-synonymous changes, and transitions and transversions were calculated from the nucleotide alignments. All substitutions were compiled and plotted against the time value for each respective virus, i.e. the number of days from the date the ancestral virus was isolated. To calculate the rate of change, linear regressions were plotted on the charts to create *y*; values were quoted as changes per site per year (Figure 5.2 and Equation 5.1). Linear regressions were performed on the sets of data for M1 and M2, for nucleotide, amino acid, synonymous and non-synonymous changes, and for H3N2, H1N1, and amantadine-resistant and -sensitive data (Figures 5.3 to 5.6; Appendix 1 to 20). The *y* values obtained from the linear regressions were used to calculate the rate of change for all of the above data sets.

FIGURE 5.2 Estimation of the rate of evolution using linear regression



Red circles represent a hypothetical data set of changes within a protein or gene over a set period of time compared to an ancestral strain (time = 0; changes = 0). A linear regression performed on the data points results produces a "y" value that can then be used to calculate the rate of change using Equation 5.1.

EQUATION 5.1 Calculation of the rate of change of gene/protein per site per year

$$\frac{a \times b}{c} = \text{Rate of Change per site per year}$$

Where a = regression value "y"; b = number of days in year (365); c = number of sites in gene or protein.

FIGURE 5.3 Rate of change of M1 in H3N2 viruses

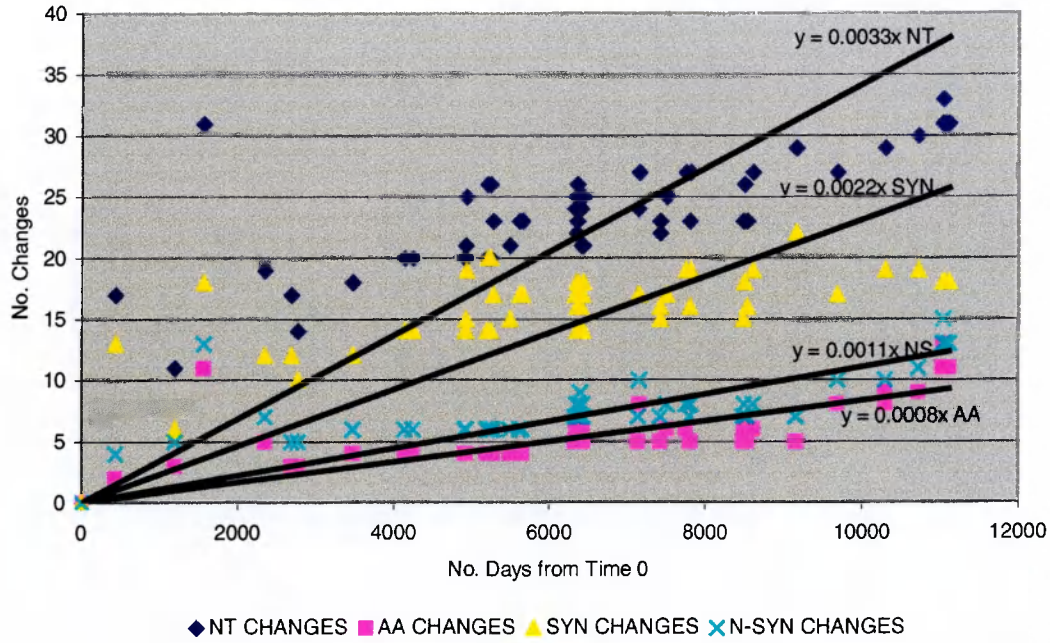


FIGURE 5.4 Rate of change of M1 in H1N1 viruses

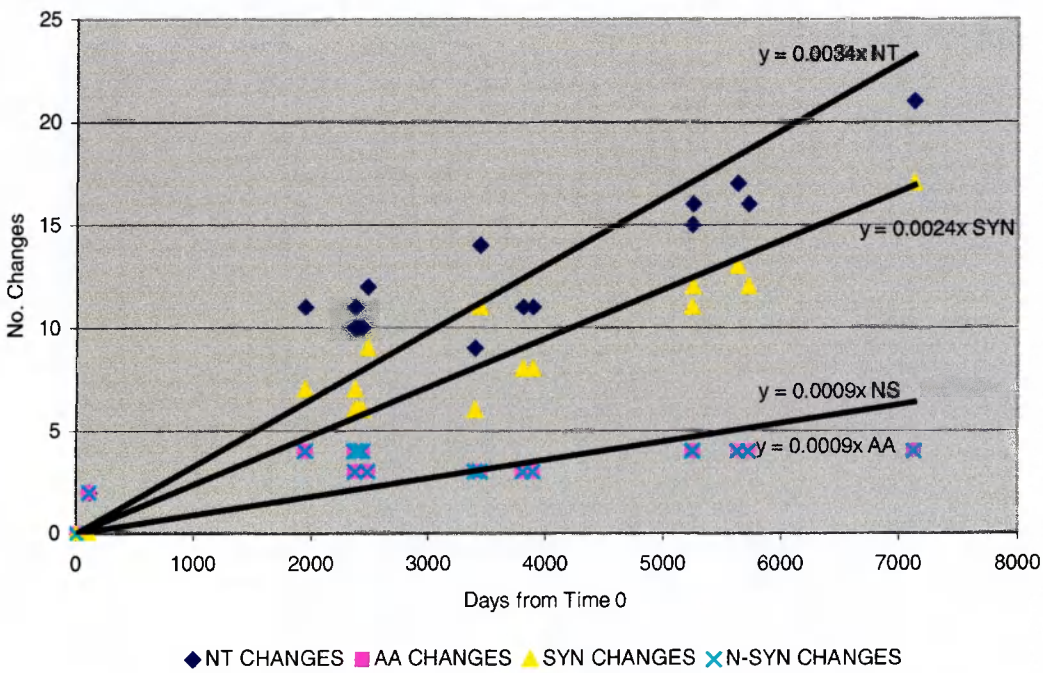


FIGURE 5.5 Rate of change of M2 in H3N2 viruses

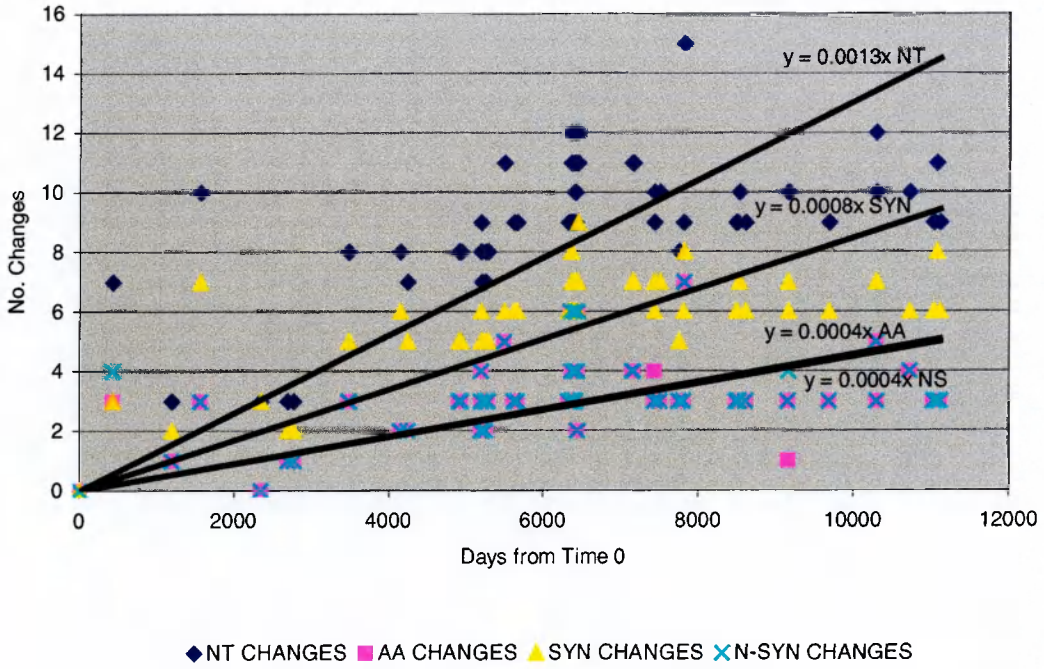
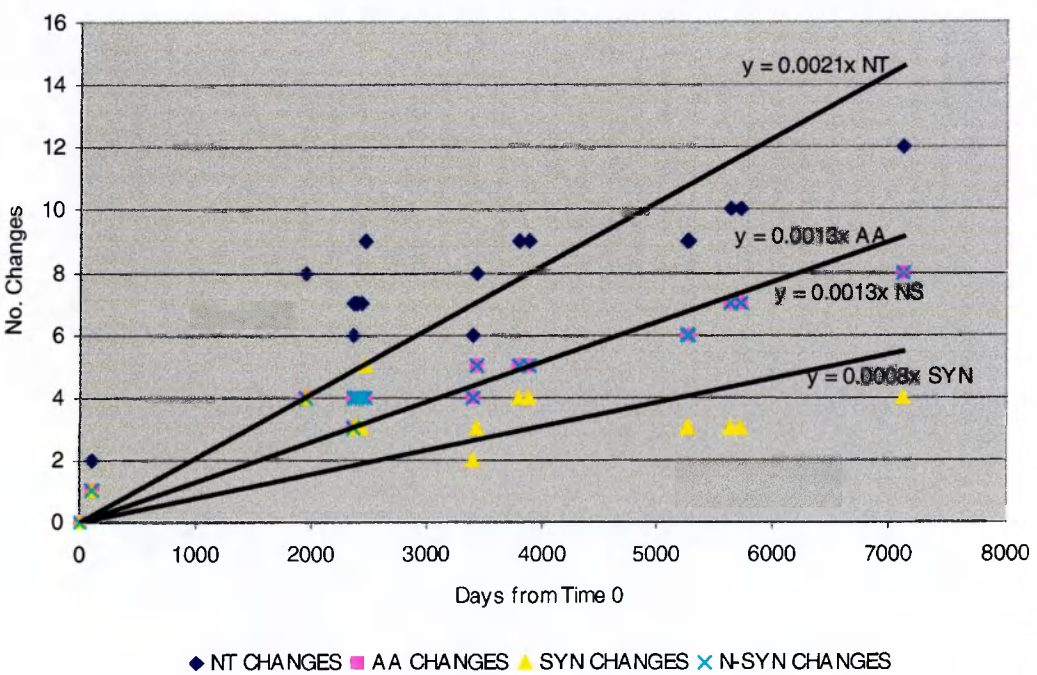


FIGURE 5.6 Rate of change of M2 in H1N1 viruses



Initially, data were analysed for the M1 and M2 proteins in total. In order to make a comparison of the rate of evolutionary change of the M1 and M2 proteins, a mean value of the rate of change of both proteins was calculated (Table 5.2). It was apparent that the rate of change (nucleotides and amino acids) of the M2 protein was greater than the M1 protein, more significantly in respect to the rate of change of amino acids per site per year which evolved at approximately two-fold that of M1. Interestingly, the rate of synonymous changes was higher in M1, and the rate of non-synonymous changes higher in M2. The significance of these findings will be discussed in section 5.4. The total rate of change for M1 and M2 was calculated for H3N2 and H1N1 subtypes using regression data from Figures 5.3 to 5.6 (Table 5.3). In general, it appeared that the evolution of M1 between H3N2 and H1N1 viruses was similar compared to M2 where H1N1 viruses displayed a greater rate of change than H3N2. To further explore this, the data sets were analysed separately for each protein.

TABLE 5.2 Comparison of the mean total rate of change of M1 and M2 of influenza A viruses

	Total nt/site/yr	**Total aa/site/yr**	**Total syn/site/yr**	**Total n-syn/site/yr**
M1	1.7×10^{-3}	1.4×10^{-3}	1.2×10^{-3}	0.5×10^{-3}
M2	2.1×10^{-3}	3.3×10^{-3}	1.0×10^{-3}	1.1×10^{-3}

TABLE 5.3 Total rate of change of influenza A M1 and M2

	Virus	**Ts:Tv (number)**	**Ts:Tv (ratio)**	**Total nt/site/yr**	**Total aa/site/yr**	**Total syn/site/yr**	**Total n-syn/site/yr**
M1	H3N2	1129:404	3:1	1.6×10^{-3}	1.2×10^{-3}	1.1×10^{-3}	0.5×10^{-3}
	H1N1	208:62	3:1	1.6×10^{-3}	1.3×10^{-3}	1.2×10^{-3}	0.4×10^{-3}
M2	H3N2	552:34	16:1	1.6×10^{-3}	1.5×10^{-3}	1.0×10^{-3}	0.5×10^{-3}
	H1N1	129:36	4:1	2.6×10^{-3}	4.9×10^{-3}	1.0×10^{-3}	1.6×10^{-3}

Ts – transition; Tv – transversion.

The rate of change of the M1 protein was calculated for each subtype and drug genotype (Table 5.4). Between H3N2 and H1N1 viruses there was no apparent variation observed in the rate of change of the protein for both nucleotides and amino acids; similarly, the Ts:Tv ratio was almost identical for all viruses. When the amantadine-resistant and -sensitive viruses of both H3N2 and H1N1 subtypes were analysed no significant trends were observed. The H3N2 resistant viruses had lower values overall than H3N2 sensitive viruses, and the H1N1 resistant viruses had higher values than the H1N1 sensitive viruses. Therefore, it did not appear that there were any significant differences in the rate of change of the M1 protein between either antigenic subtypes or drug phenotypes of the influenza A viruses tested.

TABLE 5.4 Total rate of change of influenza A M1

Virus	N	Ts:Tv (number)	Ts:Tv (ratio)	Totai nt/site/yr	Total aa/site/yr	Totai syn/site/yr	Totai n-syn/site/yr
H3N2 R	37	678:239	3:1	1.7×10^{-3}	1.2×10^{-3}	1.2×10^{-3}	0.5×10^{-3}
H3N2 S	27	451:465	3:1	2.0×10^{-3}	1.6×10^{-3}	1.3×10^{-3}	0.7×10^{-3}
H1N1 R	10	109:26	4:1	1.6×10^{-3}	1.6×10^{-3}	1.1×10^{-3}	0.5×10^{-3}
H1N1 S	11	99:36	3:1	1.5×10^{-3}	1.2×10^{-3}	1.2×10^{-3}	0.4×10^{-3}

N – number of viruses; R – amantadine-resistant; S – amantadine-sensitive; Ts – transition; Tv – transversion.

The rate of change for the total M2 protein of the influenza A viruses tested in this study was analysed for H3N2, H1N1, amantadine-resistant, and amantadine-sensitive viruses (Table 5.5). Overall it was found that the H1N1 viruses had a higher rate of change for both nucleotide and amino acid when compared to H3N2 viruses. This was most pronounced for amino acid changes where the rate was almost three-fold higher. It was also found that within the H3N2 group, the amantadine-resistant viruses had a higher rate of change than the viruses that were sensitive. When the total data were analysed for each functional domain of M2, it became apparent that there were several trends that could be identified. The rate of change within the extracellular domain (nucleotide residues 1-72) was extremely low for all viruses tested and, in some instances, there had been no change observed in nucleotides or amino acids between the viruses over the study period. The transmembrane domain (residues 73-129) demonstrated a greater rate of evolutionary change compared to the extracellular region. Overall, within this domain the H1N1 viruses appeared to have been evolving at a higher rate than the H3N2 viruses, and again the rate was generally higher for resistant viruses. The cytoplasmic domain (residues 130-291) was found to have the highest rate of change of all the functional domains of M2. H1N1 viruses again demonstrated higher rates of change but between amantadine-resistant and sensitive viruses of the same antigenic subtype, there appeared to be no differences in the rate of amino acid changes per year.

TABLE 5.5 Rate of change of influenza A M2 proteins

	Virus Type	N	Ts:Tv (number)	Ts:Tv (ratio)	Total nt/site/yr	Total aa/site/yr	Total syn/site/yr	Total nsyn/site/yr
TOTAL	H3N2 R	37	349:17	21:1	1.8×10^{-3}	1.9×10^{-3}	1.1×10^{-3}	0.6×10^{-3}
	H3N2 S	27	203:17	12:1	1.5×10^{-3}	1.5×10^{-3}	1.0×10^{-3}	0.5×10^{-3}
	H1N1 R	10	59:15	4:1	2.5×10^{-3}	4.9×10^{-3}	1.0×10^{-3}	1.6×10^{-3}
	H1N1 S	11	70:21	3:1	2.6×10^{-3}	4.9×10^{-3}	1.0×10^{-3}	1.6×10^{-3}
EXTACELLULAR	H3N2 R	37	40:0	40:0	1.0×10^{-3}	0.2×10^{-3}	0.5×10^{-3}	0.05×10^{-3}
	H3N2 S	27	20:0	29:0	0.5×10^{-3}	0.2×10^{-3}	0.5×10^{-3}	0.05×10^{-3}
	H1N1 R	10	0:0	0:0	0.0×10^{-3}	0.0×10^{-3}	0.0×10^{-3}	0.0×10^{-3}
	H1N1 S	11	4:2	2:1	0.5×10^{-3}	0.8×10^{-3}	0.3×10^{-3}	0.3×10^{-3}
TRANSMEMBRANE	H3N2 R	37	41:13	3:1	1.3×10^{-3}	1.2×10^{-3}	0.6×10^{-3}	0.4×10^{-3}
	H3N2 S	27	5:13	1:2	0.6×10^{-3}	0.2×10^{-3}	0.6×10^{-3}	0.05×10^{-3}
	H1N1 R	10	8:11	1:1	3.8×10^{-3}	5.8×10^{-3}	1.3×10^{-3}	1.9×10^{-3}
	H1N1 S	11	9:9	1:1	3.2×10^{-3}	5.8×10^{-3}	1.9×10^{-3}	1.9×10^{-3}
CYTOPLASMIC	H3N2 R	37	268:4	67:1	2.3×10^{-3}	2.7×10^{-3}	1.4×10^{-3}	0.9×10^{-3}
	H3N2 S	27	169:4	42:1	2.0×10^{-3}	2.7×10^{-3}	1.1×10^{-3}	0.9×10^{-3}
	H1N1 R	10	51:4	13:1	3.4×10^{-3}	6.1×10^{-3}	1.1×10^{-3}	2.0×10^{-3}
	H1N1 S	11	57:10	6:1	3.4×10^{-3}	6.1×10^{-3}	1.1×10^{-3}	2.0×10^{-3}

The rate of change is shown for both nucleotide and amino acid expressed for total M2 and each respective functional domain (extracellular, transmembrane and cytoplasmic) illustrated in the left-hand column of the table. N – number of viruses; R – amantadine-resistant; S – amantadine-sensitive; Ts – transition; Tv – transversion.

It was important to analyse the rates of synonymous and non-synonymous changes within the sequences of the viruses tested. For total M2, the rate of synonymous changes was similar between both H3N2 and H1N1, and amantadine-resistant and -sensitive viruses (Table 5.5). H1N1 viruses had a higher rate of non-synonymous changes per year, approximately three-fold greater. Within the extracellular domain, rates of synonymous and non-synonymous changes were extremely low. Within the transmembrane domain, H1N1 viruses had higher rates of synonymous and non-synonymous changes than H3N2 viruses. There was an interesting relationship within the transmembrane domain, where H3N2 resistant viruses had identical rates of synonymous changes to sensitive viruses, but had a higher rate of non-synonymous changes: conversely, H1N1 resistant viruses had an identical rate of non-synonymous changes to sensitive viruses but had a lower rate of synonymous changes. Within the cytoplasmic domain, H1N1 viruses had a higher rate of non-synonymous changes that H3N2 viruses but the synonymous changes were the same for all viruses with the exception of H3N2 resistant viruses that were higher.

The ratio of transitions to transversions (Ts:Tv) was calculated (Table 5.5). This figure was quoted as the actual number of Ts and Tv mutations found, and also as an adjusted ratio, to compensate for the different sizes of each domain, thereby allowing a direct comparison. For total M2, there was a much higher Ts:Tv ratio found within the H3N2 viruses compared to the H1N1 group (Table 5.5). Interestingly, within the transmembrane region the Ts:Tv ratio was much lower and in some cases there was a greater number of Tv than Ts mutations. The highest Ts:Tv ratio was found in the cytoplasmic region where again, the ratio was significantly higher in H3N2 viruses.

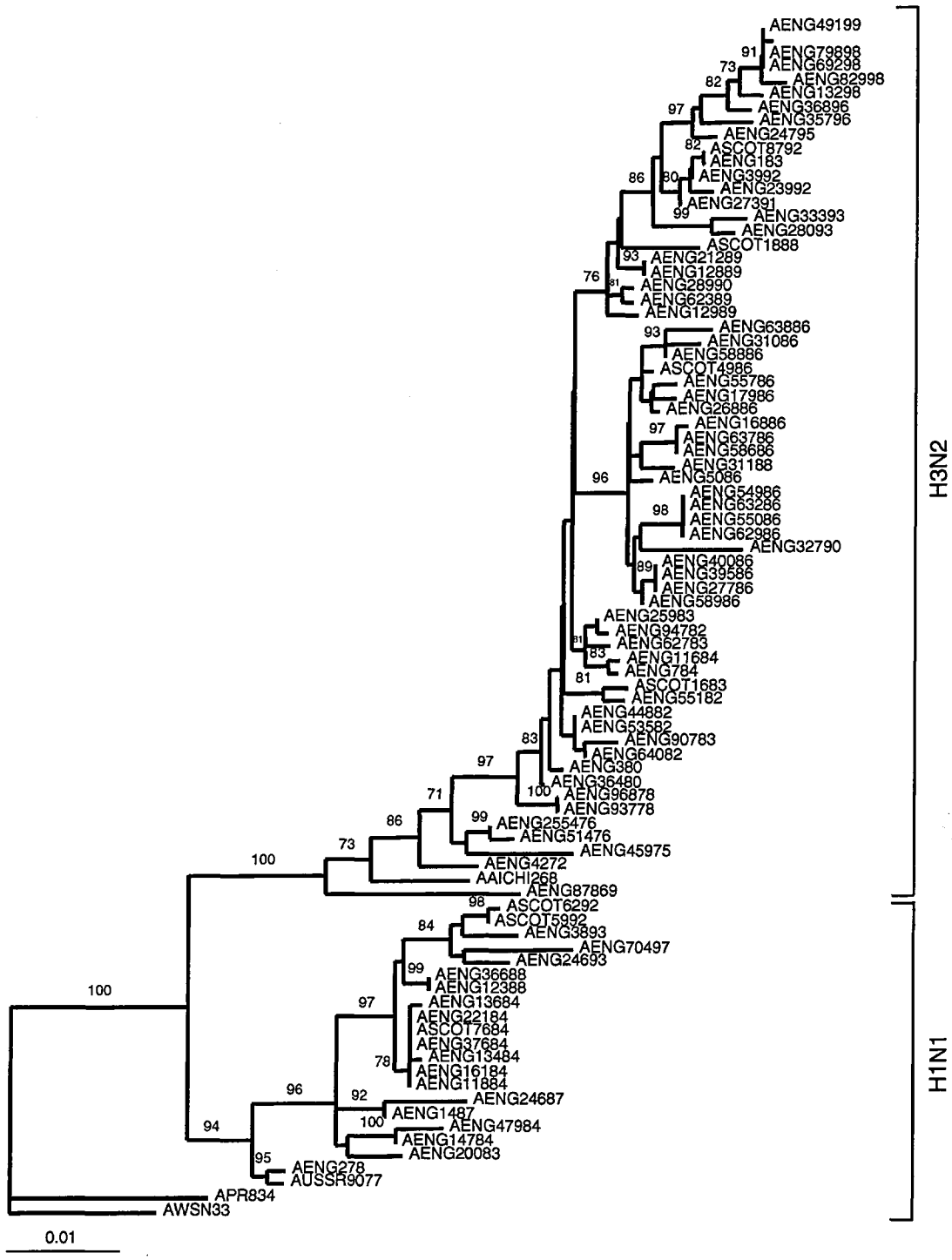
Therefore, from analysing the data it was apparent that there were domain-, subtype- and drug phenotype-specific differences in the rate of change of the M2 protein in the influenza A viruses tested. However, the rate of change within M1 was relatively constant for all variables analysed.

5.3.2 Phylogenetic Analysis of M2 Originating from Resistant Strains

Alignments constructed for the estimation of the rate of evolution of the matrix genes were modified and used to construct phylogenetic trees. Trees were constructed from nucleotide sequences for each subtype and phenotype (Figures 5.7 to 5.13; Appendix 21 to 24). Initially, a sequence alignment of the entire matrix gene was prepared. The matrix gene spans 1027 nucleotide residues but several sequences in the alignment were slightly shorter than this due to poor quality reads at the extremities of the primers. In order to produce a phylogenetic tree all sequences had to be of identical length, therefore they were cropped to the length of the shortest sequence, which produced a shortened sequence of 972 residues to be used for phylogenetic analysis. Trees for M, M1 and M2 were initially constructed containing H3N2, H1N1, resistant, and sensitive viruses (Figures 5.7 to 5.9). Bootstrap values were calculated and values greater than 70 were superimposed onto the phylograms. Analysis of the resulting trees revealed two distinct lineages within each phylogram, containing the two antigenic subtypes. The trees were relatively similar in shape suggesting that there were no major differences between each gene in respect to their evolutionary pathways, although it did appear that the tree depicting M2 evolution was slightly "flatter" over the more recent isolates. Another interesting observation was the appearance of two distinct branches within the M1 H3N2 lineage (Figure 5.8). This was most pronounced within the M1 tree but was also evident within the M2 tree to a lesser extent (Figure 5.9).

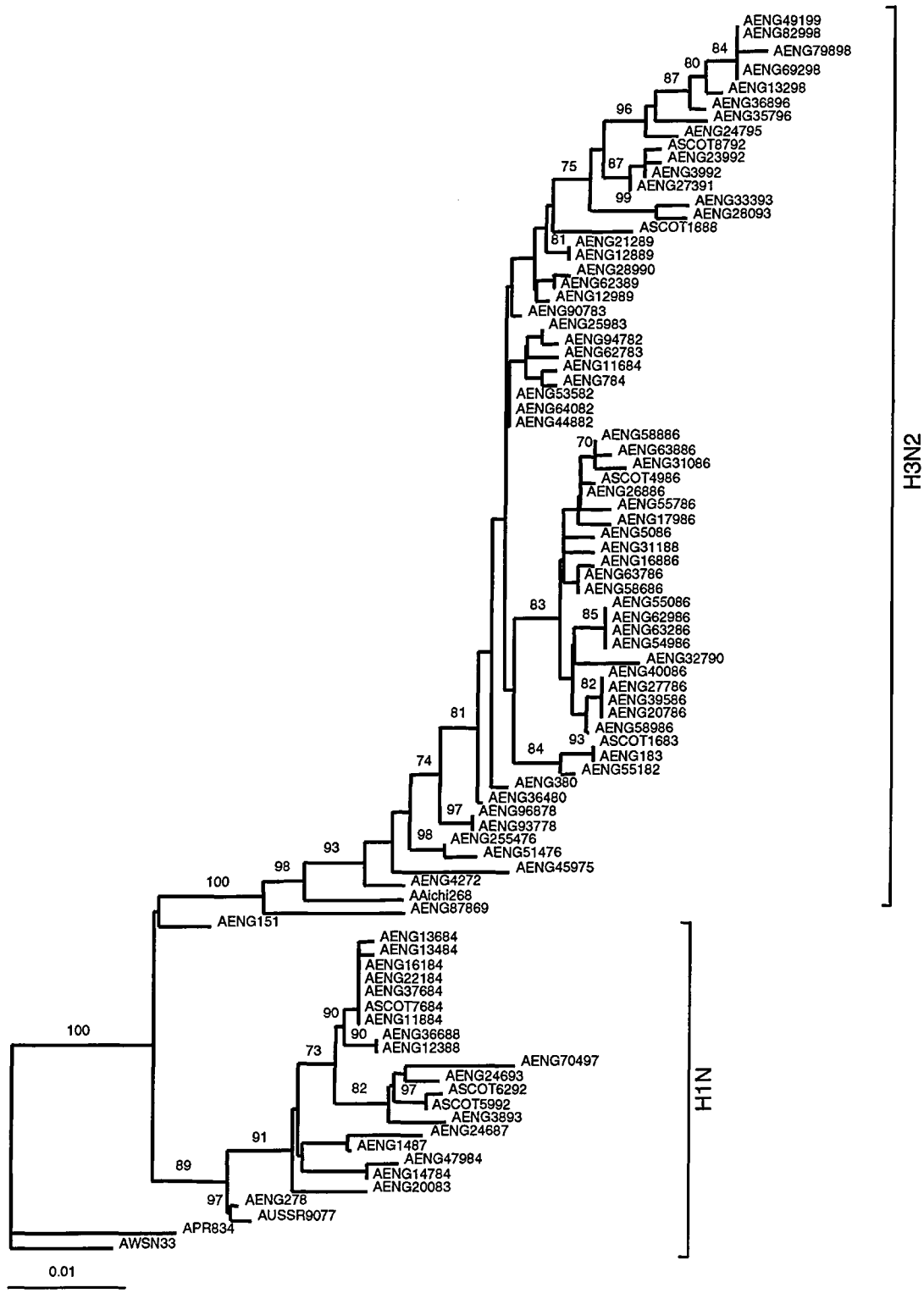
Further evidence for this phenomenon was obtained when the trees were separated into subtypes for each gene (Figures 5.10 to 5.13). The phylogeny of M1 and M2 originating from human H3N2 strains indicates two distinct groups of viruses that have evolved along separate branches. It was also apparent that the branch that separated from the main trunk in both the M1 and M2 H3N2 trees, contained a high proportion of amantadine-resistant viruses (Figures 5.10 and 5.12).

FIGURE 5.7 Phylogenetic analysis of matrix protein of influenza A viruses 1968-1999



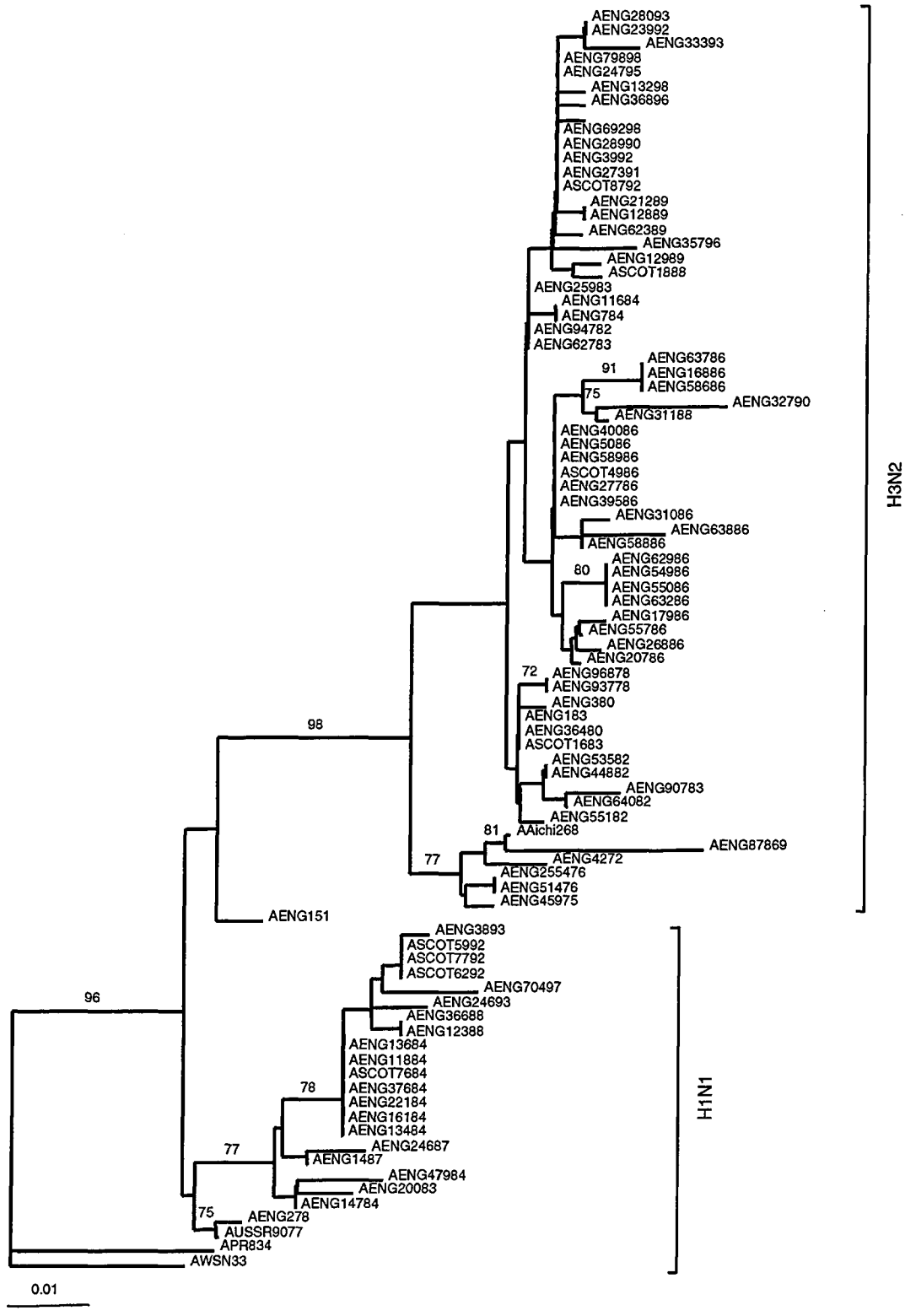
Trees were constructed using all H3N2 and H1N1 viruses used in the present study. Branches containing H3N2 and H1N1 subtypes have been labelled. Trees were bootstrapped and values greater than 70 displayed on relevant branches.

FIGURE 5.8 Phylogenetic analysis of M1 nucleotide sequence of influenza A viruses 1968-1999



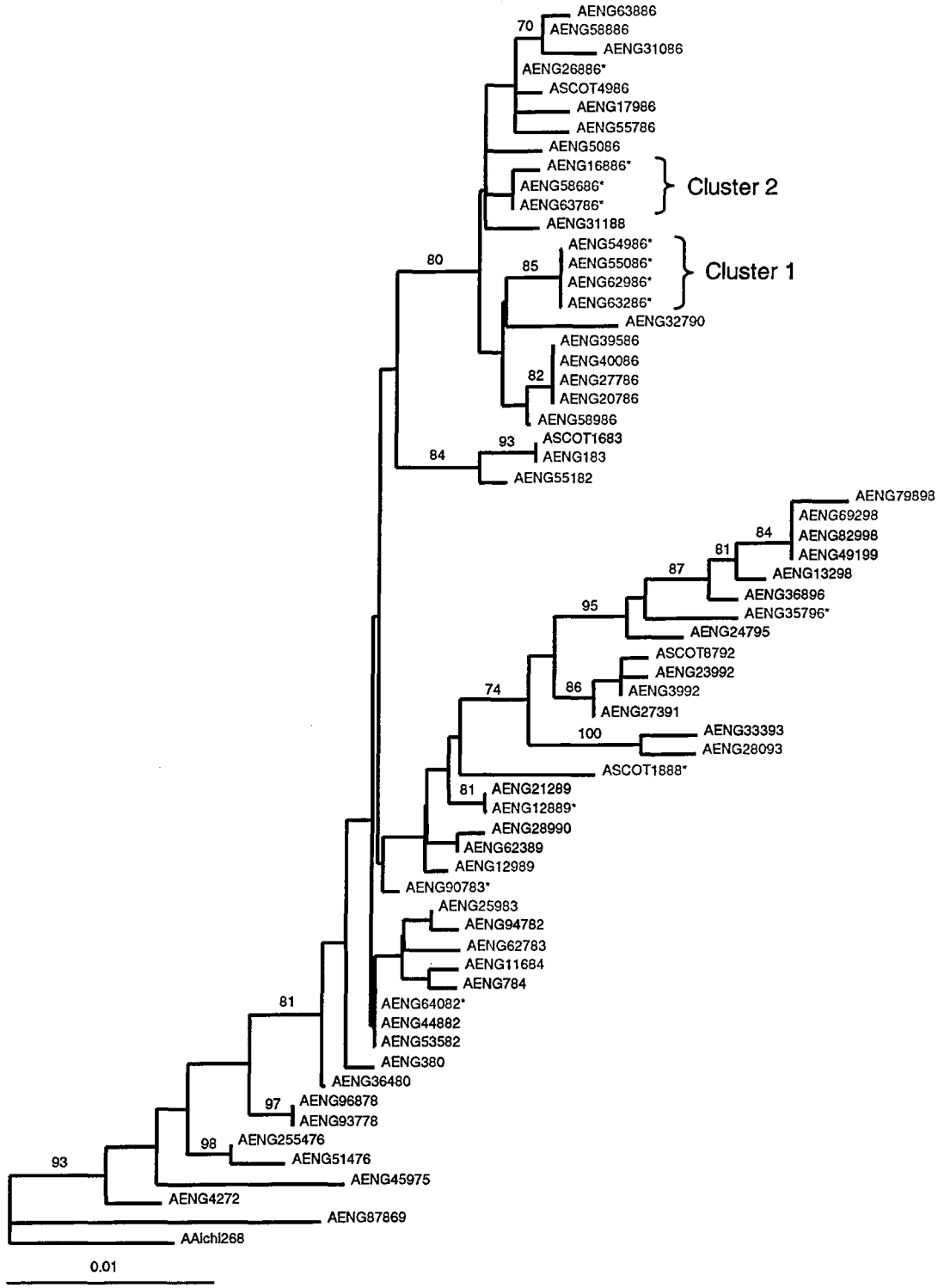
Trees were constructed using all H3N2 and H1N1 viruses used in the present study. Branches containing H3N2 and H1N1 subtypes have been labelled. Trees were bootstrapped and values greater than 70 displayed on relevant branches.

FIGURE 5.9 Phylogenetic analysis of M2 nucleotide sequence of influenza A viruses 1968-1999



Trees were constructed using all H3N2 and H1N1 viruses used in the present study. Branches containing H3N2 and H1N1 subtypes have been labelled. Trees were bootstrapped and values greater than 70 displayed on relevant branches.

FIGURE 5.10 Phylogenetic analysis of M1 nucleotide sequence from H3N2 viruses

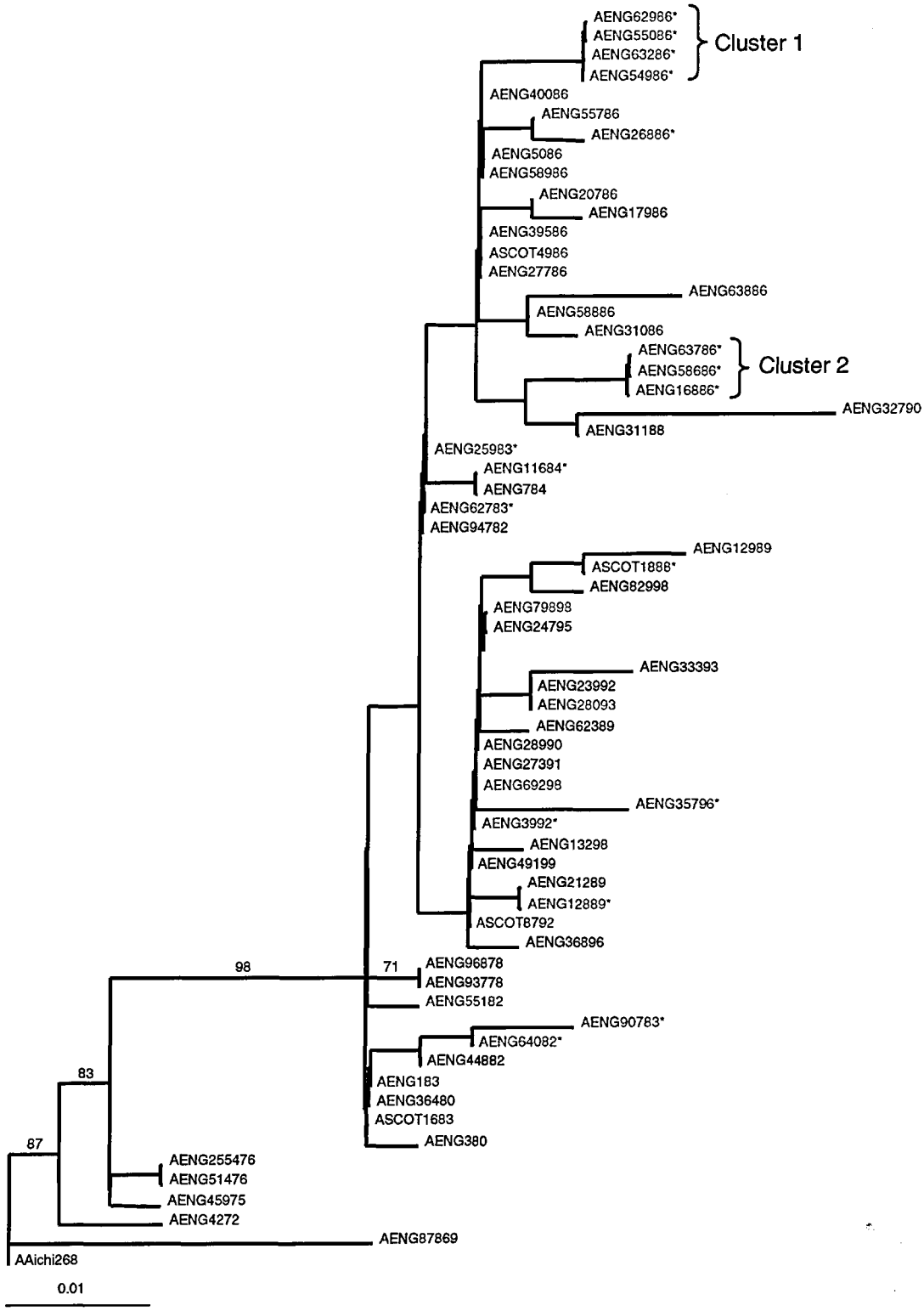


Trees were bootstrapped and values greater that 70 displayed on relevant branches. Viruses phenotypically resistant to amantadine are highlighted in red; those shown to carry mutation within the M2 transmembrane region are marked with a red asterisk. Two groups of viruses isolated from outbreak cases (Clusters 1 and 2) have been highlighted.

FIGURE 5.11 Phylogenetic analysis of M1 nucleotide sequence from H1N1 viruses

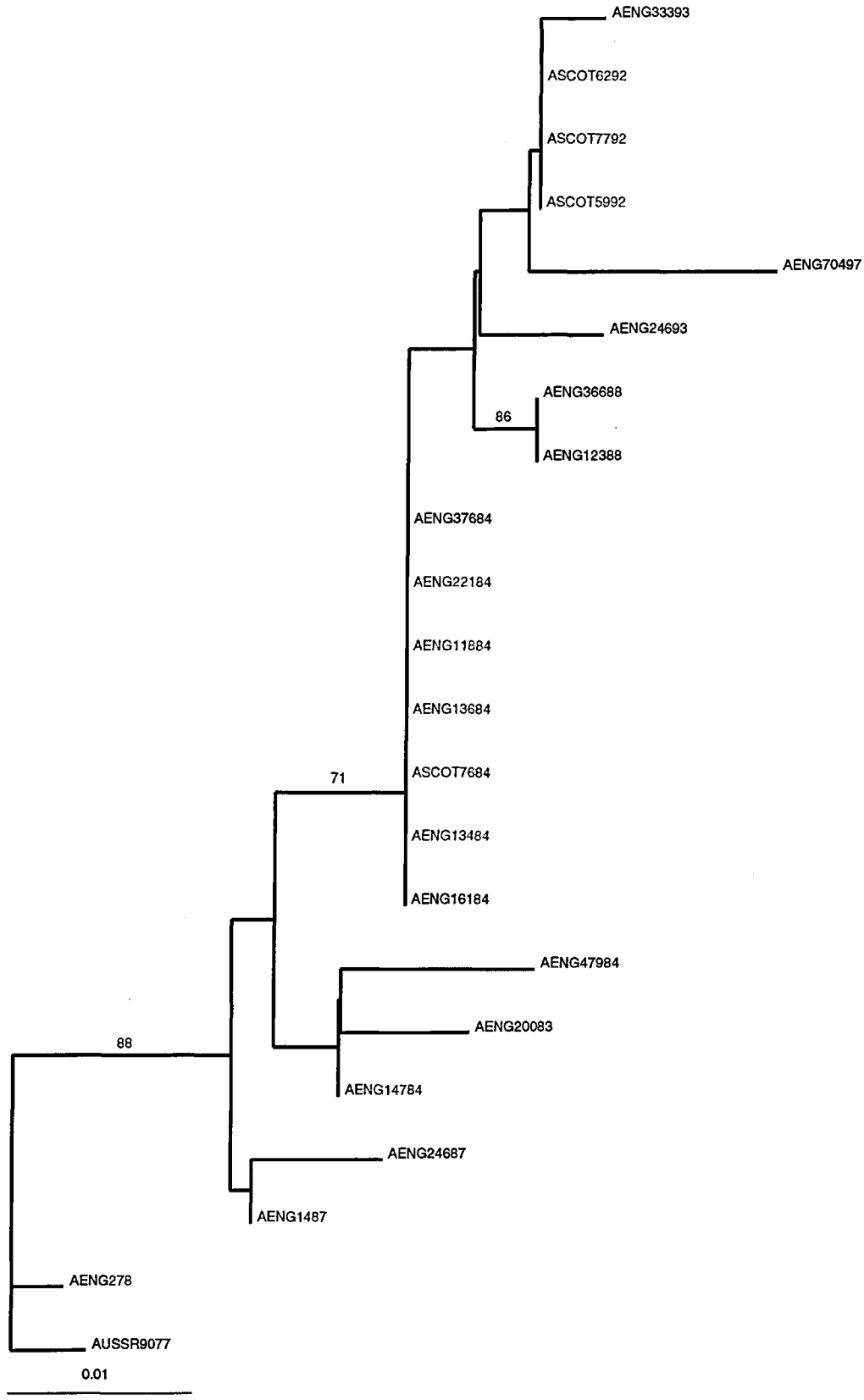
Trees were bootstrapped and values greater than 70 displayed on relevant branches. Viruses that were phenotypically resistant to amantadine are highlighted in red.

FIGURE 5.12 Phylogenetic analysis of M2 nucleotide sequence from H3N2 viruses



Trees were bootstrapped and values greater than 70 displayed on relevant branches. Viruses phenotypically resistant to amantadine are highlighted in red; those shown to carry mutation within the M2 transmembrane region are marked with a red asterisk. Two groups of viruses isolated from outbreak cases (Clusters 1 and 2) have been highlighted.

FIGURE 5.13 Phylogenetic analysis of M2 nucleotide sequence from H1N1 viruses



Trees were bootstrapped and values greater than 70 displayed on relevant branches. Viruses that were phenotypically resistant to amantadine are highlighted in red.

5.4 Discussion

5.4.1 Rate of Evolution of M1 and M2

Analysis of the results demonstrated that overall, M2 evolved at a faster rate than M1 at both the nucleotide (2.1×10^{-3} and 1.7×10^{-3} changes per site per year respectively) and the amino acid level (3.3×10^{-3} and 1.4×10^{-3} changes per site per year respectively). These figures appear to be higher than values from comparable studies (175) but demonstrate the same difference in evolutionary rate between the two proteins.

The results from this work demonstrate that within the M2 protein, the three functional domains appear to evolve at different rates. The least evolutionary change was observed within the extracellular domain; this supports data presented in Chapter 4 illustrating the extremely conserved nature of this region at both the nucleotide and amino acid level (Figures 4.9 to 4.13; pages 119, 124, 125, 129 and 130). it is possible therefore, that a functional constraint on the extracellular domain has resulted in the same sequence being conserved over the study period. The function of the extracellular domain remains unclear but it is thought to interact with other membrane proteins and facilitate their incorporation into progeny virions during the replication cycle (272). It would appear that the extracellular domain is not able to carry a significant number of mutations, most probably because they are deleterious to the function of the protein, preventing the formation of infectious particles.

The rate of change within the M2 protein transmembrane domain was shown to be higher in amantadine-resistant strains for both H3N2 and H1N1 viruses. It seemed that the most likely reason for this result was the occurrence of mutations within the transmembrane domain of the viruses causing resistance. It was also interesting to note that the ratio of Ts:Tv mutations was very low within the transmembrane domain. The reason for this observation was unclear but it is possible that the M2 transmembrane domain has greater "plasticity" than other regions of the protein and is therefore able to accommodate mutations that are not readily selected when occurring in other domains. The M2 ion channel can accommodate mutations that confer amantadine-resistance and alter the structure of the pore region. The resulting virus can remain infectious, however, which

demonstrates how the domain can retain its function whilst accommodating structure-altering changes. It is possible that the Tv changes are more likely to alter the structure or chemical composition of a protein due to the nature of the mutation. Therefore this again demonstrates that the transmembrane domain can withstand the effects of Tv mutations. It is likely that the benefit of the plasticity of the transmembrane domain is that the virus can gain resistance to amantadine through these mutations thereby providing the virus with evolutionary selection advantage under certain conditions. Such structure-changing mutations in the other domains may not provide such selective advantages and are therefore not fixed.

The cytoplasmic domain was found to be evolving at a rate higher than the other two functional domains of M2. The rate of amino acid change was the same for both amantadine-resistant and -sensitive viruses of H3N2 and H1N1 subtype. The rate of nucleotide change was the same for H1N1 viruses. However, the H3N2 resistant viruses rate of change was higher than H3N2 sensitive viruses. In the case of the H1N1 viruses, this provided evidence that there were no mutations occurring within the cytoplasmic domain that may have conferred resistance to the virus. Therefore, it could be concluded that the cytoplasmic domain did not have any direct control over the amantadine susceptibility of the H1N1 viruses. Although the nucleotide change rate of the H3N2 resistant viruses was higher than the sensitive H3N2 viruses, it appeared that the cause of this was a higher rate of synonymous changes i.e. silent mutations. These changes would not have affected the amino acid sequence of the protein and therefore it was concluded that within the H3N2 subtype viruses, the cytoplasmic domain did not have any role in determining the susceptibility of the viruses to amantadine.

To explain the differences observed between M1 and M2 in respect to evolutionary change, it is postulated that the structure/function relationships associated with each protein were involved. M2 is classified as a surface antigen; at least 18 residues of the extracellular domain are present at the cell/membrane surface (206). Although only a minor component of the influenza A virion (411), M2 is expressed abundantly at the membrane surface of virus-infected cells and therefore represents a target for the immune response (206). The presence of anti-M2 antibodies suggests that during infection, humoral immunity to the M2 protein could provide a role in the protection against influenza A (29, 362, 411).

The immunogenic property of M2 has led to research that has investigated the vaccination potential by using purified M2 preparations to produce heterotypic immunity against influenza A (100, 331). It could be hypothesised that the production of anti-M2 antibodies would increase the selective pressure on M2, forcing changes within its amino acid sequence in order for it to evade detection.

A reason why the three domains appeared to evolve at different rates may have been due to the host immune response. The M2 protein is positioned with the extracellular domain presenting at both the virion and infected cell surface. This would suggest that the primary immunological epitopes on the protein would be situated within this domain. Studies with an anti-M2 monoclonal antibody 14C2 demonstrated that the 14C2 antibody-binding site was located within the extracellular terminus of M2; the epitope was further defined to involve residues 11 and 14 (411). Although the antibody-binding site of 14C2 lies within the extracellular domain, the residues surrounding the epitope are highly conserved (Chapter 4; (175, 412). If the virus was to select variants carrying mutations, which would enable it to escape detection by antibody, then it could be hypothesised that the extracellular domain should be more variable; the results from this study and others demonstrate that this is not true. Characterisation of 14C2 resistant variants revealed that mutations within the cytoplasmic domain conferred resistance to M2 antibody inhibition (410). Although the effect of the antibody on M2 is not clear, it is likely that the binding of the antibody to the extracellular domain causes a conformational change in the cytoplasmic tail region. Antibody-resistant variants containing mutations within the tail may prevent this change occurring. This theory can therefore be linked with the above results; it was postulated that immune pressure has caused the formation of viruses with mutations within the cytoplasmic domain rendering them resistant to antibody. Results from Chapter 4 confirm this; mutations at amino acid position 78 were found (Figures 4.11 and 4.13; pages 125 and 130) which have been correlated to antibody resistance (410). Therefore, the increased rate of evolution within the cytoplasmic tail may be a direct consequence of the pressure exerted on the extracellular domain of the protein. Another study conducted with anti-M2 mouse sera demonstrated its reactivity to different synthetic peptides produced representing the entire M2 sequence (331). This study demonstrated that the highest serum antibody titre was raised against a peptide representing amino acid residues 79-97. The second highest titre was achieved with a peptide representing residues 2-18. This was

evidence that although epitopes for the laboratory-produced monoclonal antibody (14C2) were found within the extracellular domain, the primary epitopes for this naturally-occurring antibody were located within the cytoplasmic region. This supported the results found in this study and was an additional explanation for the increased rate of change found within the cytoplasmic region of M2. An antibody binding to this region would force the selection of mutants allowing the virus to escape the activity of the antibody. The study by Slepushkin *et al.* (1995) also provided evidence that relatively high antibody titres were produced against synthetic peptides representing the N-terminal region of the M2 protein supporting the 14C2 work by Zebedee and Lamb (1989) (331, 410).

The location of M2 at the infected cell/viral membrane surface would suggest that the evolution of this gene would be in excess of reported estimates and more like those reported for the other influenza A surface glycoproteins (Table 5.1). Conversely it has been suggested that constraints placed upon M2 might restrict the rate of change within the gene. This might be as a result of the bicistronic nature of the M gene; M1 and M2 are transcribed from gene segment seven and they share a sequence of nine amino acids at the NH_3-terminal end (204). It has been proposed that M1 has several important functions within the replication cycle of the virus, and mutations causing amino acid changes within the protein may be detrimental to the survival of the virus. Therefore, the relatively low rate of evolution of M1 might exert an evolutionary constraint on the rate of change of M2 in order to retain M1 as a functional protein. The results demonstrated that the rate of M1 was lower than that of M2 (1.4×10^{-3} and 3.3×10^{-3} amino acid changes per site per year, respectively). M1 is an internal virus protein located between the lipoprotein and the RNP (413). Due to the location of the protein within the virion, there is far less potential for immune pressure to be exerted compared to M2, a possible explanation for observed results. However, there has been evidence to show that M1 is recognised by human cytotoxic T lymphocytes and therefore may be subjected to some degree of immune pressure (116, 176, 296). This might correlate to the low levels of evolutionary change observed within M1 over the study period.

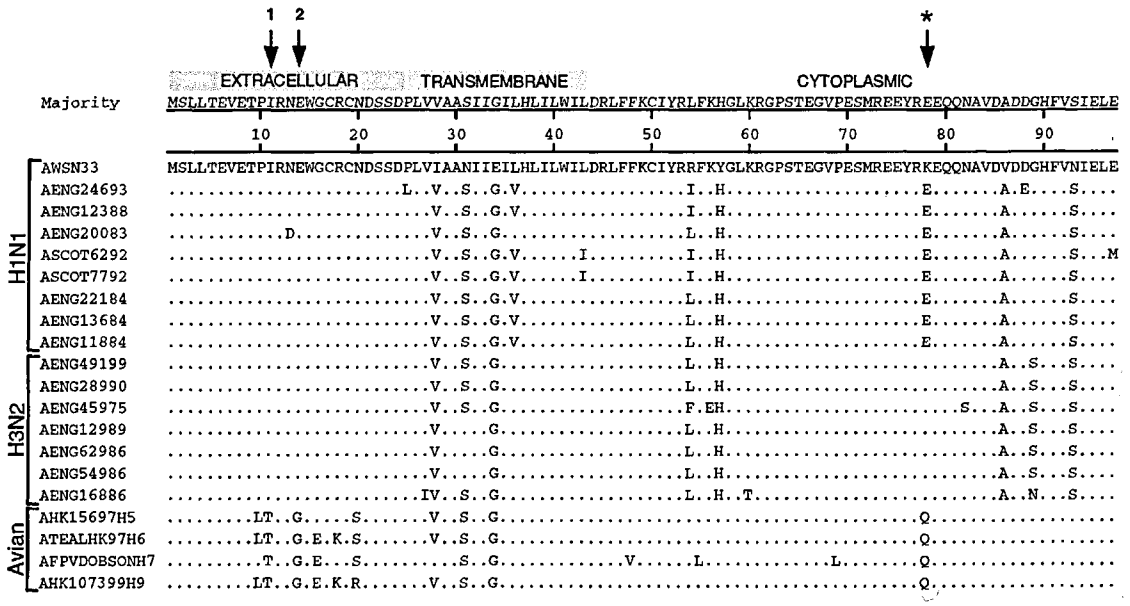
Results demonstrated that overall, M2 isolated from H1N1 viruses evolved at higher rates than H3N2 viruses. This was an interesting observation; comparisons of the rate of change of other influenza A genes between the H3N2 and H1N1

subtypes are limited to the HA1 gene where it is reported that compared to H1N1, H3N2 viruses evolved at elevated rates (59). The observed difference could be attributed to the smaller sample size of the set of H1N1 viruses simply causing a bias in the results. There were approximately three H3N2 viruses tested for every H1N1 virus, however this ratio represented the circulation of the viruses within the community over the study period. Alternatively, it could be that M1 and M2 of H1N1 viruses may have been more closely related to another species lineage other than human. If the matrix genes were more avian in character than human, when in the human host, the viruses would demonstrate an increased rate of change in an attempt to adapt to the host. To investigate this idea, a sample of human H1N1 and H3N2 and avian viruses were analysed phylogenetically (data not shown). The results were inconclusive; the H1N1 lineage did not appear to be closer to avian species than the human H3N2 viruses within the phylogenetic tree. The H1N1 viruses were not significantly more avian in character and therefore the increased rate of change observed is likely to be caused by another factor.

The genetic characterisation of both amantadine-resistant and -sensitive strains revealed a trend among H1N1 viruses (Chapter 4, Figures 4.11 and 4.13, pages 125 and 130). The majority (95%) of the H1N1 viruses sequenced contained a Glu residue at position 78 within the cytoplasmic tail. As previously discussed, this mutation had been associated with conferring resistance to the M2 antibody 14C2. Therefore, the assumption could be made that a large proportion of the H1N1 viruses in this study would be resistant to the activity of anti-M2 antibody. The 14C2 antibody has been shown to reduce the growth of certain influenza virus strains (411). Viruses resistant to the antibody would have the potential to grow and therefore replicate at an increased rate. It might be hypothesised that without the selective pressure from the immune system, and increased viral growth rates, the matrix genes from these antibody-resistant viruses would evolve at a higher rate than antibody-sensitive strains. This hypothesis correlated with the data presented in this study; all H1N1 viruses, irrespective of drug phenotype or genotype had a greater rate of change over the study period than H3N2 viruses. An amino acid alignment of human and avian viruses was created to see whether the Glu residue at position 78 was also found in avian viruses (Figure 5.14). A selection of H1N1 and H3N2 viruses from this study, and four avian viruses of different subtype were used. Sequences were aligned to A/WSN/33, this represented the earliest prototype strain for the viruses. As previously shown, the

H1N1 viruses all carried a Glu at position 78 except A/WSN/33, which in common with the H3N2 viruses had a Lys residue (Figure 5.14). Interestingly, M2 from avian strains contained a Lys78Gln mutation. This suggested that these avian strains had a mutation rendering them resistant to the effects of the 14C2 antibody, similar to postulations made about the H1N1 viruses in this study.

FIGURE 5.14 Amino acid alignment of M2 protein of human and avian influenza A strains



Amino acid alignments of human and avian influenza A M2. Sequences were aligned to the prototype strain A/WSN/33. Arrows 1 and 2 indicate the residues thought to be involved in the binding of anti-M2 antibodies. The asterisk highlights residue 78 within the cytoplasmic domain, thought to confer resistance to MAb 14C2 activity (410).

Another observation made was that within the extracellular domain of the avian strains, there were several differences in sequence compared to the human viruses. In particular, there were differences between residues 11 and 14, the avian strains carried Ile11Thr and Glu14Gly mutations (Figure 5.14). These residues have previously been demonstrated to comprise the epitope for the 14C2 antibody (410). Therefore, these avian strains appeared to have mutations at both sites for antibody binding and at positions within the cytoplasmic region that have been shown to confer resistance to the antibody. The implications of these findings are interesting. Although only four avian viruses were selected for this analysis, two represented subtypes (H5N1 and H9N2) that have been the cause of recent

avian to human transmission of influenza (48, 129). The hypothesis can be made that the anti-M2 antibody 14C2 has less antiviral activity against these avian strains. This could be construed as an additional explanation for the increased pathogenicity and virulence demonstrated by these viruses when they cross the species barrier into humans. The M2 protein is highly conserved amongst influenza A viruses and therefore may offer cross-protection to the host from novel viruses (175, 410). If these avian strains carried mutations allowing them to evade this form of immunity then it could result in an increased potential for severe disease.

In critique of the work performed here, it was recognised that there were some weaknesses associated with the estimation of the rates of evolution for particular genes. This was especially apparent when the transmembrane region of M2 was analysed. This region is relatively conserved and the occurrence of mutations is predominantly associated with viruses acquiring amantadine-resistance (118, 142, 175, 412). Due to the lack of evidence for transmission of resistant viruses in the community presented in this study (Chapter 3), it might be hypothesised that under normal conditions, without the selective pressure of drug, the genotype of the virus will revert to sensitive. This aspect of the virus presents problems for calculating the rate of change. If over a period of time, the only mutations occurring within the M2 transmembrane region were as a result of amantadine-resistance and therefore not fixed, then there would be no overall accumulation of mutations. This makes the calculation of the rate of change difficult to interpret because the resulting figure does not give an accurate picture of what is actually happening. The evolution of other influenza genes also seems to involve the appearance and disappearance of mutations, but there is also a proportion of mutations that become fixed within the virus population that results in the overall accumulation of mutations over time.

Another critical aspect of the work was the small sample size within some data sets. For example, when analysing the resistant H1N1 viruses, there were only ten data points to plot (Appendix 9, page 290). When performing a linear regression on this small data set it was apparent that certain data points, that in a larger set of data would not have been so influential, might have biased the best line of fit. However, the methods used to calculate the rates of change were similar to those

used in other studies and therefore it was thought that those methods should be employed here to enable the comparison of results.

5.4.2 Phylogenetic Analysis

Phylogenetic analysis of the M, M1 and M2 nucleotide sequence revealed that there were two subtype-specific lineages of the matrix genes, H3N2 and H1N1. Further analysis of the H3N2 lineage revealed genetic divergence of M1 into two distinct branches. The branch that had separated from the main trunk of the tree contained viruses isolated between 1982 and 1990 although the majority (80%) were isolated during the 1985/86 influenza season; the main trunk of the tree has continued to evolve until present day. Interestingly, the branch that separated from the main trunk was almost entirely composed (88%) of amantadine-resistant (phenotypic or genotypic) strains. It was hypothesised that from the emergence of the H3N2 subtype in 1968, one lineage of M1 had evolved until approximately 1982 when the genetic divergence occurred and the second branch separated. This could have been as the result of the appearance of a variant that circulated in the population. This variant could have had a lower susceptibility to amantadine, or had the potential to be replicated and transmitted when carrying a resistant genotype. This would have resulted in the appearance and circulation of amantadine-resistant viruses over the period that the variant was present in the population. Evidence from the phylogeny suggests that the first amantadine-resistant strains appeared during the 1982/83 season at which point the variant emerged and circulated.

Analysis of the phylogeny of the M2 H3N2 tree showed that similar to M1, there was a divergence of the gene into two distinct branches. However, it was found that there were differences between the separated branches of M1 and M2. Each branch contained a similar number of viruses but they were not identical.

The presence of the branch containing mainly amantadine-resistant strains supported conclusions that were drawn in Chapters 3 and 4. It was found previously that a large proportion of the viruses that were resistant by phenotypic assays did not carry any mutations within the M2 transmembrane domain. It could be that the presence of quasispecies within each virus isolate tested may have resulted in the amplification of a genome from a sensitive genotype. In a highly resistant strain this would not be as common because the balance of resistant

genotypes would outweigh the sensitive genotypes. However, in a virus of low resistance a higher proportion of sensitive genomes may have been present, increasing the chance that the M2 sequence produced would not contain any mutations within the transmembrane domain. Analysis of the population dynamics of the viruses that were phenotypically resistant but lacked mutations within the M2 transmembrane region would support this theory. Two approaches could be made; initially, viruses of interest could be titrated in a plaque reduction assay in the presence and absence of amantadine. Plaques formed in the presence of drug could be picked, biologically cloned and sequenced to determine the genotype of the virus particles contained within the plaque. Alternatively, using a different cloning method, gene segment seven of the virus could be amplified and transformed into a suitable bacterial host. Selection of a number of colonies, and amplification and sequencing of the gene segment seven contained within each colony would give an estimate of the heterogeneity of the gene sequence within the virus sampled.

The phylogeny of the viruses tested demonstrated that viruses resistant by phenotype and genotype were grouped together in the second main branch. This indicated that these viruses were very similar in respect of M1 and M2. Therefore it was concluded that this was further evidence of the presence of quasispecies within individual virus isolates that had biased the results. It was interesting to note that within the cluster of amantadine-resistant strains there were also several sensitive viruses interspersed. This suggested that these viruses were very similar to the resistant strains. It would be interesting to repeat the testing of these viruses to see whether the original phenotypic and genotypic testing had not detected their resistance. If this reveals that they were actually resistant this would demonstrate how phylogenetic analysis could indicate the drug susceptibility of viruses.

Suspected outbreaks of amantadine-resistant viruses as indicated in Chapter 3 were identified within the trees. There were two small groups of viruses identified within the M1 and M2 trees that could be correlated with viruses suspected of originating from outbreaks (Figures 5.10 and 5.12). The first group (A/ENG/629/86, A/ENG/550/86, A/ENG/632/86 and A/ENG/549/86) all carried a Ser31Asp mutation within the M2 transmembrane domain and epidemiological data indicated that they were originally isolated from Guildford. Therefore, the clustering of these viruses within the trees was further evidence that the viruses were isolated from an

outbreak of an amantadine-resistant strain. The other small group of viruses (A/ENG/637/86, A/ENG/586/86 and A/ENG/168/86) all similarly contained a Ser31Asp mutation within the M2 transmembrane domain. Again, phylogenetic analysis provided evidence of the emergence and possible transmission of amantadine-resistant viruses.

Overall, the appearance of the phylogenetic trees suggested that the main trunk of the phylograms, containing a majority of amantadine-sensitive viruses was evolving at a relatively steady rate. This was especially true of the matrix and M1. The branch containing resistant viruses appeared to be more stationary, i.e. there was no evolution occurring in these viruses. This may have been because the majority of the viruses contained within the branch were isolated in the 1985/6 influenza season and therefore, over such a short period of time it would be difficult to ascertain the evolution of the genes. Alternatively, the resistant phenotype and genotype might have put an evolutionary constraint on the viruses. This might have been evidence that these viruses, carrying mutations within M2, were at a selective disadvantage to the sensitive wild-type viruses and therefore, once emerged, could not survive within the population for any significant period of time.

5.4.3 Concluding Remarks

In summary, the work from this Chapter estimated the relative rates of evolution of the gene segment seven from influenza A viruses illustrating that the different proteins transcribed from gene segment seven were undergoing different evolutionary rates of change. It was hypothesised that these observations were as a consequence of the different levels of selective pressure exerted on each respective protein. Phylogenetic analysis of the M1 and M2 separated viruses into subtype-specific lineages with some clustering of amantadine-resistant strains into separate branches.

Chapter 6

Study of a Persistent Influenza Infection in an Immunocompromised Host

6.1 Introduction

Natural infections of human influenza are generally self-limiting in healthy adults. Following the introduction of virus into the host, a series of events stimulate the immune response, which eventually prevails in eliminating the invading pathogen. In certain situations, where the immune system of the host has been depleted or suppressed, the virus has an opportunity to prolong replication and therefore the period of infection, often resulting in increased severity of disease or persistence of infection. It has been commonly reported that one of the groups most at risk from complications arising from influenza infection are the immunocompromised where the immune system has been suppressed either through drug therapy (e.g. cancer or organ transplants) or disease (e.g. human immunodeficiency virus; HIV).

The consequences of a deficient immune system in the immunocompromised host can have numerous effects on the response to infection. Some forms of chemotherapy have been reported to reduce mucosal integrity and possibly IgA antibody levels within the upper respiratory tract; these two factors represent one of the first lines of natural defence against infection. Several forms of immune deficiency result in reduced serum immunoglobulin levels, T cell function and neutrophil numbers. The overall severity of immune deficiency has been shown to have a direct consequence on the frequency and severity of infections acquired (223, 311). Both severe and mild immune deficiency in immunocompromised patients account for a higher frequency of infection and greater risk of death when compared to immunocompetent hosts.

Influenza infections in immunocompromised children have been poorly documented. Feldman *et al.* (1977) studied twenty children and young adults with cancer; each patient was receiving immunosuppressive therapy (95). Although the symptoms presented by the patients were not unusual, the clinical course of disease lasted twice as long (two weeks) as in the general population. This study also found that the rate of complications arising in the patients was low, and were in fact related to secondary bacterial infections rather than as a direct result of the influenza. A similar study that followed a group of immunocompromised children with cancer determined the risk of influenza infection and severity of disease in these patients compared to a control group (183). Although there was no significant difference in duration of symptoms between the two groups, eleven per

cent of the immunocompromised children were hospitalised during their influenza illness; none of the control children were admitted (183).

Reports documenting the severity of influenza in immunocompromised patients of all age groups can be conflicting. The majority of the data suggest the influenza infections are a frequent cause of serious respiratory disease in hospitalised immunocompromised patients. This has been reported for transplant recipients (9, 239, 311) and patients with leukaemia (408), neoplastic disease (387) and cancer (95). Infections of patients in these groups can be fatal (9, 183, 274, 408). Complications arising from influenza infections in immunocompromised patients are mainly primary viral pneumonia or secondary bacterial or fungal pneumonias; the development of these complications can lead to relatively high mortality rates (388). Although the majority of data indicate that influenza is the cause of serious respiratory disease in immunocompromised patients, there are reports that suggest the contrary. A study by Ljungman *et al.* (1993) identified immunocompromised patients during the progression of two influenza seasons. They reported incidences of infection and disease to be mild and self-limiting in most immunocompromised patients but occasionally they caused severe complications (223).

General respiratory pathogens that circulate within the community are an important cause of respiratory disease in immunocompromised patients (91, 216, 385, 388). In a study of bone marrow transplant recipients during two influenza seasons, respiratory pathogens circulating within the community were diagnosed in 31 percent of recipients (388). Using a range of isolation techniques, it was determined that the infections were caused by respiratory syncytial virus (49%), influenza viruses (18%), picornaviruses (18%), parainfluenza viruses (9%) and adenoviruses (6%). The appearance and disappearance of the viruses within the patients mirrored the circulation of the viruses within the community at that time (388).

The susceptibility of immunocompromised patients to infection with community-acquired respiratory viruses means that stringent infection controls and treatments must be used to prevent or limit the severity of disease. Basic precautions to limit nosocomial transmission and spread of disease i.e. handwashing, gloving and masking, where implemented, have been shown to significantly reduce the

incidence of infection (291). Infections with RSV or PIV have the potential to cause severe lower respiratory tract disease associated with high mortality. Trials have been performed in an attempt to treat these severe infections with the nucleoside analog ribavirin. This drug has been shown to have a broad antiviral spectrum against influenza A and B viruses and some paramyxoviruses (131, 151, 167, 240, 255, 267). Against PIV infection in immunocompromised patients, intravenous ribavirin has been shown to temporarily arrest viral replication and improve clinical presentation of symptoms (151). Studies that have investigated the effect of ribavirin on immunocompromised patients infected with RSV have shown that despite treatment with the drug, mortality rates remain high (91, 215).

In addition to basic infection control procedures, two strategies exist for the prevention and treatment of influenza in immunocompromised patients; immunisation and the use of antiviral drugs. Prevention of influenza infection by vaccination is highly recommended for all at risk groups (7). Not surprisingly, the immunological response to the inactivated vaccine has been shown to be impaired during suppressive chemotherapy treatments in cancer and transplant patients (123, 269) and therefore the individual responses to vaccination are considered to be unpredictable.

The use of amantadine and rimantadine in preventing influenza A infections in immunocompetent adults has been well documented (37, 74, 149, 165). However, data for the chemoprophylactic use of these antiviral compounds in immunocompromised patients is poorly defined. A common policy for prevention of influenza infections is seasonal amantadine prophylaxis, which has been shown to be 71% effective in preventing influenza (251). Combinations of seasonal vaccination and amantadine prophylaxis during periods of influenza activity within the community can provide optimal protection against influenza for immunocompromised patients.

The use of amantadine and rimantadine for treating influenza infections in immunocompromised patients is also poorly defined. The limited data suggests that transplant patients receiving amantadine treatment recovered from infection but non-treated patients also have a high rate of rate of recovery (145). The known disadvantages of using the drugs for treatment of influenza infections, i.e. emergence of resistance and side-effects, present problems in the

immunocompromised host. The shedding of resistant viruses in immunocompromised patients has been documented (193) and shedding of virus for prolonged periods presents additional problems. Several publications have reported the shedding of influenza A and B from patients with deficient immune systems. The length of time that virus can be isolated in samples can range from days, to several months (151, 193, 304).

Immunocompromised patients diagnosed with influenza are commonly prescribed antiviral drug treatment to prevent or control disease. The net result of the treatment is the common emergence of resistant mutants. In several cases, viruses isolated from persistent infections have been characterised and mutations within the M2 transmembrane domain conferring resistance identified (90, 193). However, in the majority of cases where influenza has been isolated during or after treatment with amantadine or rimantadine, and genetic characterisation has not been possible, there is a high possibility that the viruses will show reduced susceptibility to the drugs (93).

With the recent high profile launch of influenza neuraminidase inhibitor (NI) drugs e.g. zanamivir, new hope was revived for the efficient control of influenza within the immunocompromised host. Evidence from early work and clinical trials demonstrated that unlike amantadine and rimantadine, the emergence of resistant mutants was rare both *in vitro* and *in vivo* (124, 127, 128, 242, 243). A major criticism of the NI drugs prior to launch was the lack of substantial data provided for the use of the drugs in, and the benefit provided to immunocompromised patients. Interestingly, evidence for the emergence of an influenza B mutant resistant to zanamivir was reported during the treatment of an immunocompromised patient (127). This was further evidence of the problems raised when treating influenza infections in the immunocompromised host. It would appear that the virus is able to replicate unchallenged which may give it added opportunity to develop resistance to antiviral drug therapy.

There have been numerous reports of persistent viral infections involving a range of pathogens both *in vitro* and *in vivo* (12, 13, 80, 246, 292). Persistent influenza infections have been documented *in vitro* involving different influenza types (49, 103, 115, 225). The available data show that persistent infections *in vitro* involve the maintenance of a balance between virus replication and host cell survival.

Persistent infections have been shown to involve the production of mutant virus particles or unusual patterns of protein synthesis (208, 225, 234). In particular, during a persistent infection of influenza C it was shown that viruses isolated contained single nucleotide changes and variation within separate proteins that could be functionally linked to the persistent virus phenotype (208, 234). Therefore, it seems that molecular changes within virus proteins can alter the replication of the virus in such a way that persistence within the environment becomes possible. It has also been shown that influenza viruses with the ability to initiate persistent infections are able to outcompete wild-type strains that have a "normal" replication cycle indicating that the persistent viruses are at a selective advantage (234).

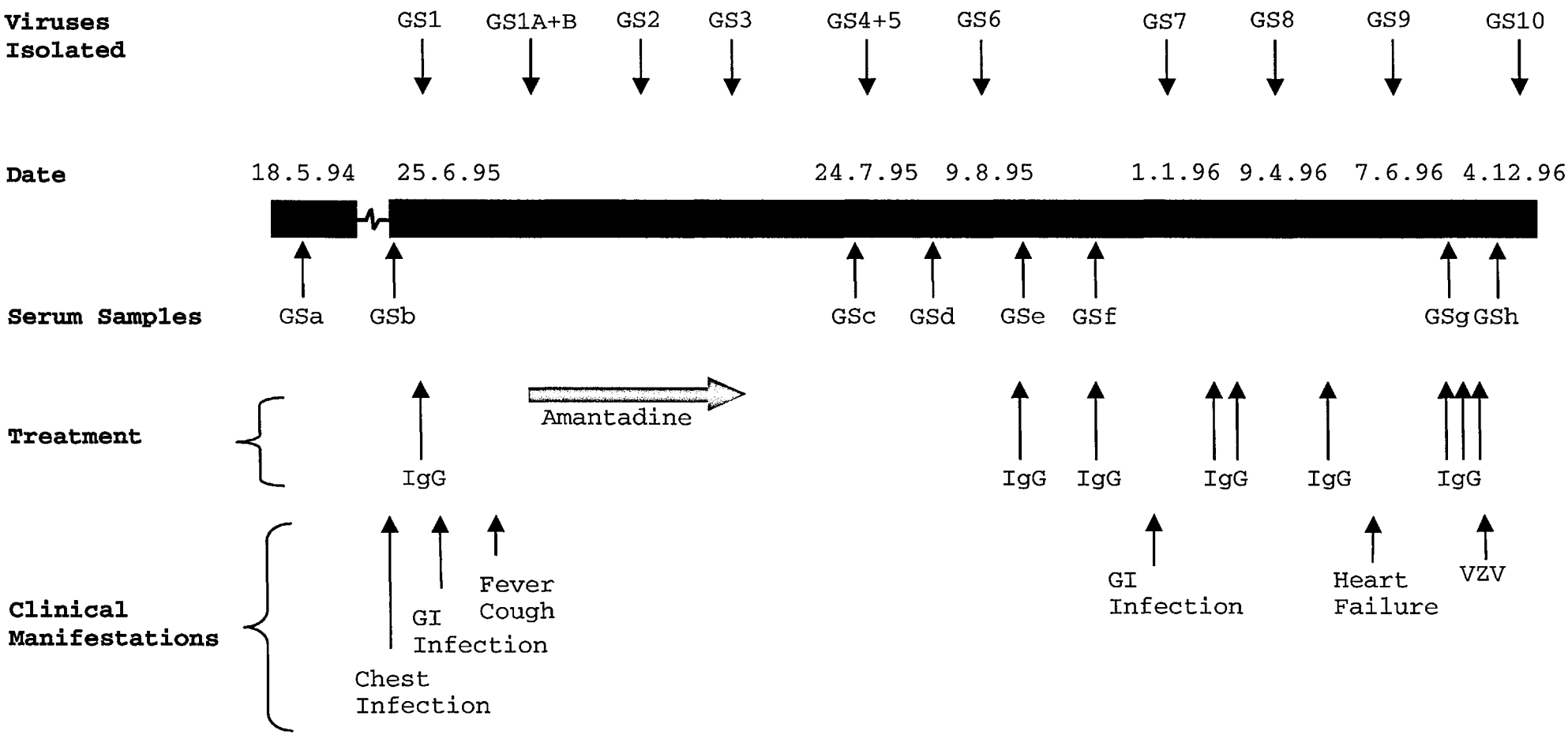
Persistent influenza infections of varying lengths within the human host have been described (90, 93, 127, 134, 193, 244, 304). Prolonged infections lasting more than a few weeks are extremely rare. The two longest recorded episodes of persistence were an influenza A H3N2 and an H1N1 infection, both of approximately ten months duration (134, 244, 304). One clinical feature associated with these cases of persistence is the infection of an immunodeficient host. This indicates that the suppressed immune response within these hosts enables the virus to replicate in the absence of any immune selective pressures and therefore prolong the period of infection.

In persistent infections of influenza, there has been evidence to demonstrate the presence of heterogeneous populations of viruses (quasispecies). With respect to drug-resistance, it has been shown that over the course of a persistent infection, viruses isolated at different stages can carry different mutations within the M2 protein conferring resistance to amantadine and rimantadine (90, 193). This suggests that within the viral quasispecies there are mixtures of variants of different drug genotype, which due to unknown pressures or variables can be selected and become the predominant species. This would account for the appearance of certain resistance mutations that can then be superseded by different genotypes. This pattern has also been documented by characterising other proteins of influenza recovered from persistently infected patients (304).

6.2 Case Study

The case studied here involved a female patient aged 13 years old (at the time of study). The patient is subsequently referred to as GS. The patient was HIV positive with a low CD4 count (18 x 10^6/l; 3%); the history of the clinical manifestations and treatments of GS are summarised in Figure 6.1. During June 1995, the diagnosis of a chest infection subsequently led to the detection of influenza A H3N2 from a nasopharengeal aspirate (NPA) by immunofluoresence; the virus was designated A/ENG/292/95 and typed by haemagglutination inhibition as A/Thess/1/95-like. A course of amantadine treatment was initiated which lasted ten days. During the amantadine treatment, several more clinical samples were collected and tested for influenza all of which were positive for influenza A (Table 6.1). Following the cessation of amantadine treatment, influenza A H3N2 continued to be isolated for a period of approximately eighteen months. Viruses isolated were propagated in MDCK cultures and stored for further analysis.

FIGURE 6.1 The progression of virus isolation and clinical manifestations of GS



Viruses and serum samples were isolated from GS over a period of approximately 18 months. The course of amantadine therapy is represented by a red arrow. GS – patient; GI – gastrointestinal; IgG – immunoglobulin; VZV – varicella zoester virus

TABLE 6.1 Summary of GS viruses

Isolate	Date of Specimen	No. Days from GS1	Specimen type
GS1	21/06/95	0	NPA
GS1A	30/06/95	9	Nose swab
GS1B	30/06/95	9	Gargle
GS2	03/07/95	12	Nose swab
GS3	10/07/95	19	Nose swab
GS4	24/07/95	33	Gargle
GS5	24/07/95	33	Nose swab
GS6	09/08/95	49	Gargle
GS7	14/12/95	156	Gargle
GS8	14/12/95	156	Gargle
GS9	25/06/96	378	Gargle
GS10	04/12/96	531	Saliva/Oral fluid

Table illustrates each sequential isolate, the date of isolation and the type of specimen from which the viruses was isolated.

6.3 Aims

The current study, where influenza A was isolated over a period of eighteen months represents the longest reported persistent infection to date and provides an invaluable opportunity to study different aspects of viral persistence in the immunocompromised host. The aims of this study were designed to investigate differences between sequential viruses isolated in respect to drug-resistance and antigenic properties. There were several objectives to the work:

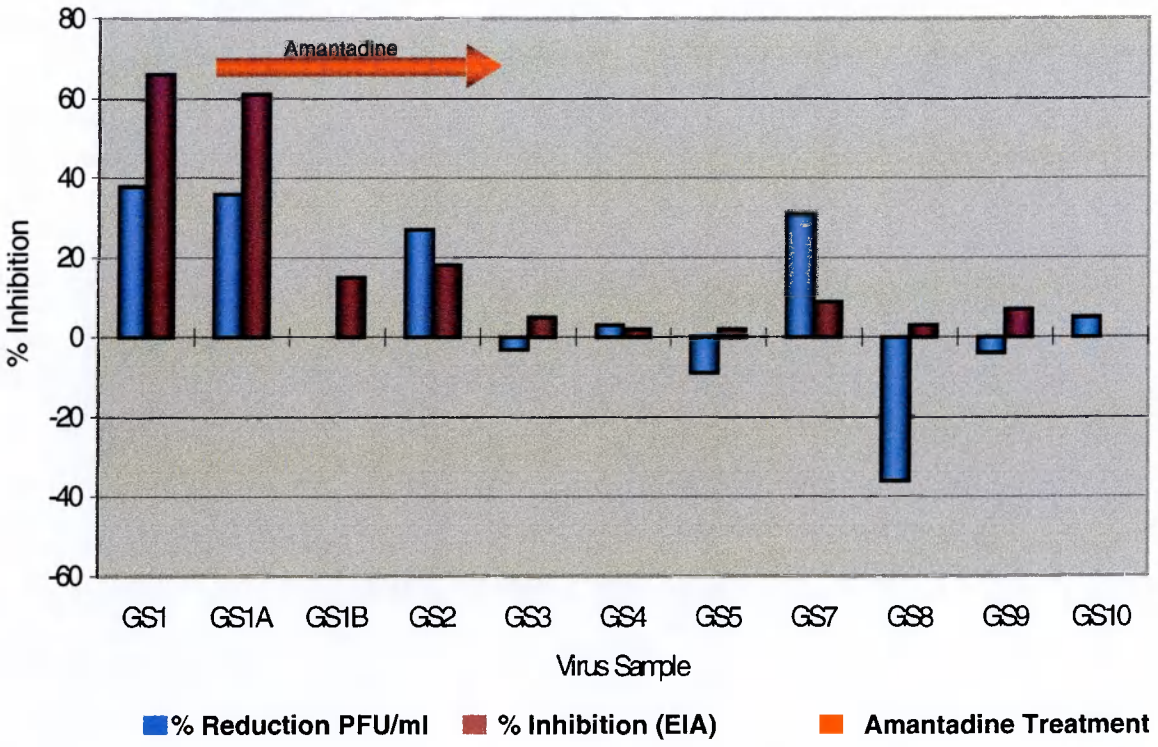
- Drug susceptibility testing of each virus including phenotypic and genotypic characterisation.
- The evaluation of antigenic properties to identify sequence changes within the sequential viruses that may provide evidence for adaptation or evolution within the host.
- The determination of immune response by GS to the initial influenza infection.
- Estimate the evolution of M1, M2 and HA1 of the GS viruses and field strains that circulated over the period of study.

6.4 Results

6.4.1 Phenotypic Analysis of GS Virus Isolates

To determine the phenotypic characteristics of each sequential virus isolated from GS, the amantadine susceptibility EIA and plaque reduction assay described in Chapter 3 were used (Figure 6.2). Each virus was grown in tissue culture prior to testing, although attempts to culture GS6 were unsuccessful meaning that this virus could not be included in any of the phenotypic testing. The initial results demonstrated that the viruses isolated after the patient's treatment with amantadine generally had reduced susceptibility to the drug compared to those isolated prior to treatment. The first two isolates, GS1 and GS1A were both considered to be amantadine-sensitive, the inhibition by EIA was greater than 60% and the reduction in PFU/ml by plaque assay was approximately 40%. Viruses isolated after this point had reduced sensitivity to the drug and were considered amantadine-resistant (Figure 6.2). The isolates GS1A and GS1B were both isolated on the same day but were different specimens originating from independent sites, a nose swab and gargle respectively. Therefore, it was interesting to note that the apparent drug susceptibility of each virus appeared to be significantly different, one sensitive and one resistant both by EIA and plaque reduction assay (Figure 6.2). Similarly, GS7 and GS8 were isolated at the same time but from different sites, and again the phenotypic characteristics of each virus differed. Although this was only apparent from the plaque reduction assay data, it appeared that GS7 had a higher sensitivity to drug than GS8, although GS8 seemed to have increased growth in the presence of amantadine. Interestingly, several other viruses (GS3, GS5, and GS9) had negative values for reduction in PFU/ml indicating that these viruses had actually increased replication rates in the presence of drug. In general, there was a good correlation between the susceptibility in EIA and plaque reduction assay; both identified resistant and sensitive viruses. The only exception was GS7, where the plaque reduction assay indicated that the virus was sensitive but results from the EIA contradicted this (Figure 6.2). Overall, the pattern of susceptibility over the range of viruses was high for the first two isolates, medium for the next two and then low for the remainder.

FIGURE 6.2 Comparison of sensitivities of GS isolates to amantadine using plaque assay and EIA



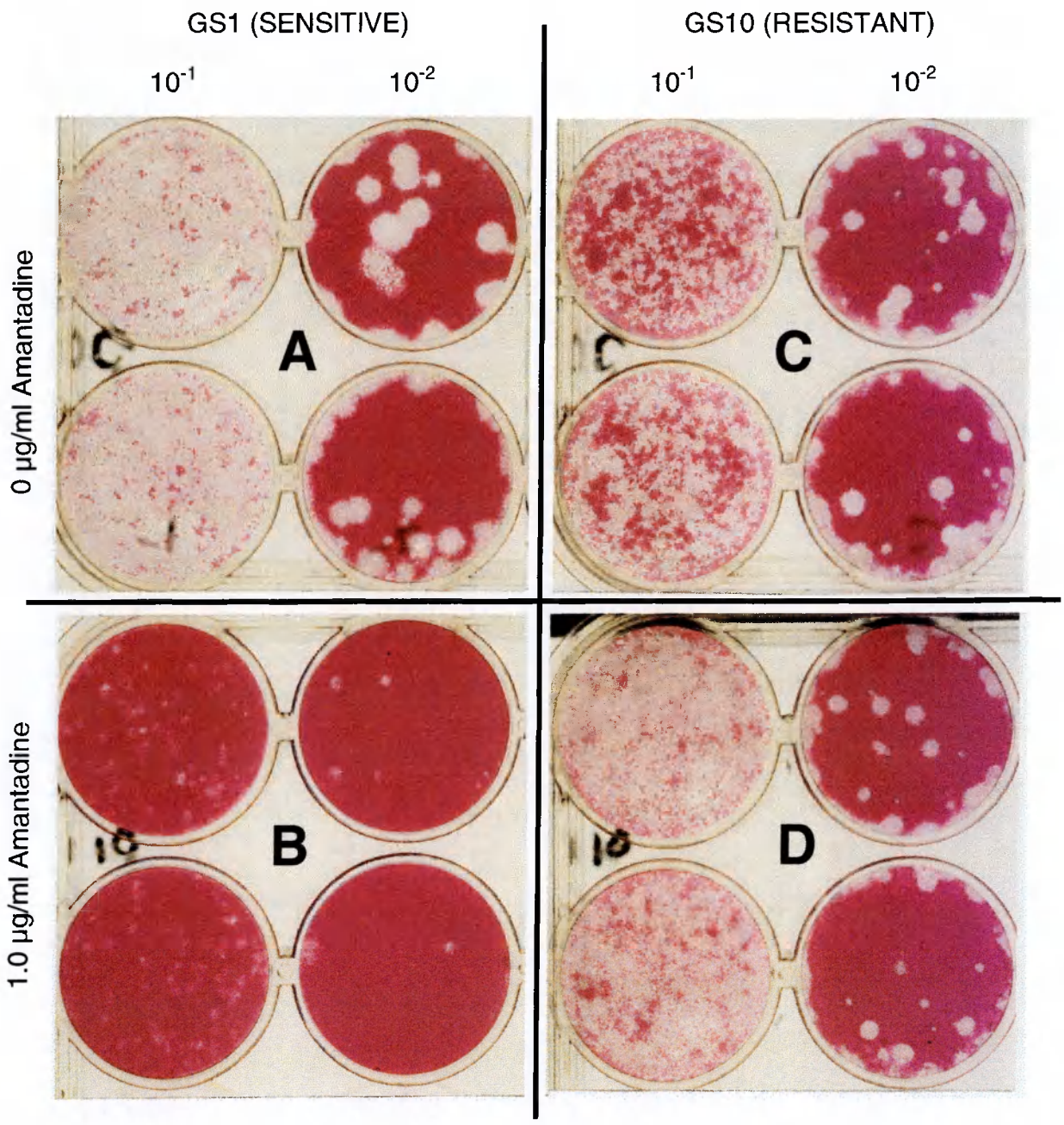
The morphology of the plaques was recorded and analysed (Table 6.2). There was a correlation between the percentage reduction in plaque size in the presence of drug and the inhibition of the viruses by EIA and plaque assay. GS1 and GS1A had plaques that showed a 62 and 46 percent reduction in size respectively when grown in the presence of amantadine. These figures were relatively large when compared to the reduction of plaques grown from other viruses isolated after amantadine treatment, these viruses generally showed a reduction in the range of –2 to 38 percent, a mean of approximately 18 percent. Therefore, it appeared that the viruses isolated prior to drug treatment had plaques that were greatly reduced in size in the presence of amantadine compared to those post treatment, which did not display such evident reductions (Figure 6.3). These results indicated some viruses had increased replication and growth rates in the presence of drug; this was a characteristic that was observed for some amantadine-resistant isolates in Chapter 3.

TABLE 6.2 Plaque morphology of sequential GS virus isolates

Virus Isolate	Isolation Date	% Red[n] PFU/ml	Plaque size (diameter, mm)		
			- Drug	+ Drug	% Reduction
GS1	21/06/95	38	2.74	1.04	62
GS1A	30/06/95	36	2	1.08	46
GS1B	30/06/95	0	2.65	2.14	20
GS2	03/07/95	27	3.08	2.2	29
GS3	10/07/95	-3	1.94	1.45	25
GS4	24/07/95	3	2.74	2	27
GS5	24/07/95	-9	2.5	2.56	-2
GS6	09/08/95	NVG*	NVG	NVG	NVG
GS7	14/12/95	31	2.7	2.29	15
GS8	14/12/95	-36	2.14	2.18	-2
GS9	25/06/96	-4	1.82	1.64	10
GS10	04/12/96	5	1.86	1.14	38

***NVG - No virus grown.**

FIGURE 6.3 Characterisation of drug-sensitive and -resistant viruses from GS



MDCK cells were infected with GS1 (A+B) and GS10 (C+D) stained at 72 hours post infection. Amantadine at 1.0 µg/ml was incorporated into overlay B+D.

6.4.2 Genotypic Characterisation of GS Virus Isolates

PCR amplification and sequencing of the GS virus isolates was performed by J.Ellis and C.Sadler of the Respiratory Virus Unit, CPHL. Amplification and sequencing of the M2 gene from the twelve sequential isolates revealed the drug genotype of each virus (Table 6.3). Sequence analysis of the transmembrane region of M2 revealed that viruses sampled prior to amantadine treatment had a sequence homogeneous to a wild-type sensitive virus. From the results it was apparent that the viruses isolated post drug treatment contained mutations within the transmembrane domain of M2. The mutations discovered all correlated to previous studies demonstrating the molecular basis for amantadine-resistance (118, 142). Another observation from resistant viruses GS1B to GS10 was that sequences from each virus were heterogeneous, i.e. there were viruses carrying different M2 mutations within the viral quasispecies (Table 6.3). The most common mutation discovered was Ser31Asn occurring in 7/12 viruses; other mutations found were Val27Ala and Ala30Thr. Three viruses were found to contain mixed populations of genotypes. The viruses GS2, GS3 and GS6 all contained mutations at sites known to confer amantadine-resistance but sequences of the sensitive wild-type viruses were also found. Interestingly, sequencing of GS3 revealed that there was a mixture of two resistant genotypes, Ala30Thr and Ser31Asn in addition to the sensitive wild-type genotype (Table 6.3).

Viruses that had been isolated on the same day were analysed for sequence homology. GS1A and GS1B were isolated from a nose swab and gargle washings respectively; both samples were collected on 30/06/95. The M2 transmembrane sequence revealed that the two viruses differed; GS1A carried a series of amino acids resembling the sensitive wild-type sequence and GS1B had a Ser31Asn mutation known to confer amantadine-resistance. GS4 and GS5 were isolated from gargle washings and a nose swab respectively and also had heterogeneous sequences. Both viruses had resistance mutations but each was different. The transmembrane sequence of GS4 had a Val27Ala mutation and GS5 a Ser31Asn mutation. The final two samples taken simultaneously were GS7 and GS8; both were gargle washings. The two viruses isolated from the samples shared complete sequence homology of the M2 transmembrane domain.

TABLE 6.3 Sequence variations in the M2 gene of isolates GS1 to GS10

Virus	Date	Amino acid position and substitution[1]					Drug
		26	27	30	31	34	Genotype[2]
Sensitive		Leu	Val	Ala	Ser	Gly	S
GS1	21/06/95	Leu	Val	Ala	Ser	Gly	S
GS1A	30/06/95	Leu	Val	Ala	Ser	Gly	S
Amantadine administered							
GS1B	30/06/95	Leu	Val	Ala	Asn	Gly	R
GS2	03/07/95	Leu	Val	Ala	Ser/Asn	Gly	M
GS3	10/07/95	Leu	Val	Ala/Thr	Ser/Asn	Gly	M
GS4	24/07/95	Leu	Ala	Ala	Ser	Gly	R
GS5	24/07/95	Leu	Val	Ala	Asn	Gly	R
GS6	09/08/95	Leu	Val	Ala	Ser/Asn	Gly	M
GS7	14/12/95	Leu	Ala	Ala	Ser	Gly	R
GS8	14/12/95	Leu	Ala	Ala	Ser	Gly	R
GS9	25/06/96	Leu	Val	Ala	Asn	Gly	R
GS10	04/12/96	Leu	Val	Ala	Asn	Gly	R

[1] Only those amino acids within the M2 transmembrane of influenza A viruses previously reported to confer amantadine-resistance are shown. [2] S - sensitive genotype; R - resistant genotype; M - mixed genotype comprising of either sensitive and resistant genotypes, or different resistant genotypes. Sequencing multiple samples of the same virus and comparing the accumulated sequences achieved data for viruses demonstrating mixed genotypes.

6.4.3 Analysis of Antigenic Properties of GS Virus Isolates

In order to determine the overall antigenicity of the GS viruses, a haemagglutination inhibition (HAI) assay was performed. A panel of polyclonal ferret antisera was prepared to include reference virus types representing the period prior to, and during, the time when the GS isolates were collected. To complete the panel, antisera were generated for the first and last GS isolates. The antisera were then reacted with the constituent viruses and the ability of the virus-serum complex to agglutinate turkey erythrocytes was assessed (Table 6.4). All of the GS viruses were included in the test but only selected strains are displayed in the results. Analysis of the results demonstrated that within the control panel of reference viruses there was good correlation between the reaction of homologous virus and antiserum, and reduced reactivity between viruses of different antigenicity i.e. antigenic drift variants. The GS viruses were isolated in the 1995/96 winter season and were antigenically related to prototype viruses isolated at the same time e.g. A/JHB/34/94 and A/Thess/1/95. The results from the HAI assay confirmed this (Table 6.4). GS10 did not react as well as GS1 with the prototypes with an observed difference in titre of up to sixteen-fold. When the results for GS1, GS8, GS9 and GS10 were analysed for reactivity to the A/JHB/34/94 and A/Thess/1/95 antisera, it appeared that the titres fell from the first virus to last. This type of pattern was evidence for antigenic drift; the gradual changes occurring in a virus over a period of type result in the virus becoming less homologous to its respective antisera resulting in the drop in reactivity titre. This suggests that over the period of persistent carriage, there was genetic drift in the viruses that resulted in antigenic variation. However, GS10 still reacted more closely to the 1995/96 prototype viruses (A/JHB/34/94 and A/Thess/1/95) than to A/Wuhan/359/95, indicating that reinfection with a different virus had not occurred.

A selection of the GS viruses was tested with the GS1 and GS10 ferret antisera (Table 6.4). The GS1 virus and homologous antiserum produced a strong end point titre (1280), however the GS10 virus combined with GS10 antiserum had a much lower titre (Table 6.4). When the selection of GS viruses was reacted with the GS1 antiserum, there was further evidence of drift occurring within these viruses. There was an eight-fold reduction in titre between the GS1 and GS10 isolates. When the GS viruses were reacted with the GS10 antiserum, the resulting titres were poor and there was no evidence of drift.

TABLE 6.4 Haemagglutination inhibition of GS virus isolates

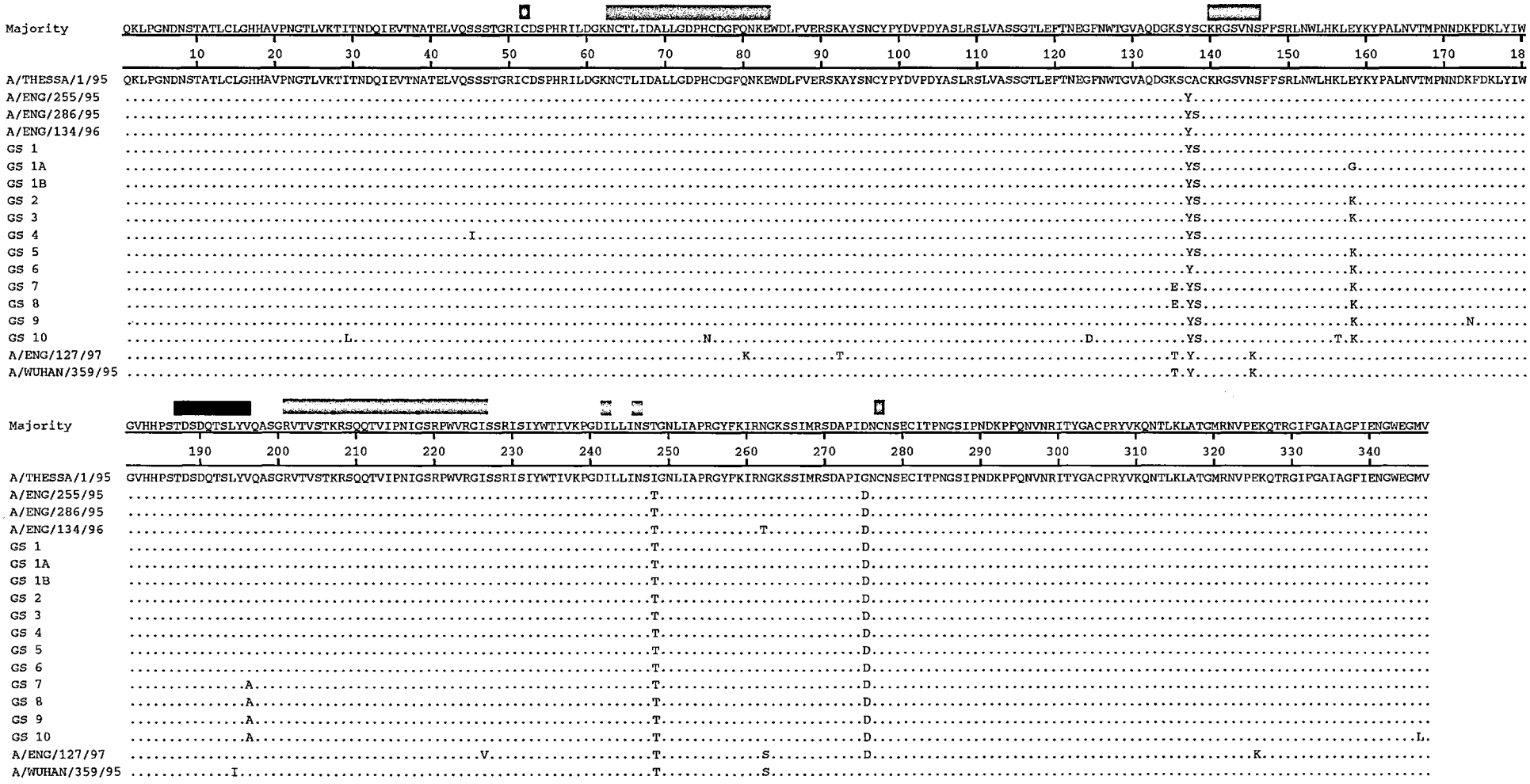
Virus	Antisera				
	GS1	GS10	A/JHB/34/94	A/Thess/1/95	A/Wuhan/359/95
GS1	**1280**	160	5120	2560	160
GS1A	ND	ND	2560	1280	80
GS1B	ND	ND	2560	1280	80
GS2	ND	ND	1280	1280	320
GS3	ND	ND	1280	640	80
GS4	ND	ND	5120	2560	80
GS5	ND	ND	640	640	80
GS7	640	80	160	640	2560
GS8	640	320	2560	2560	160
GS9	160	160	2560	1280	80
GS10	160	**160**	320	320	40
A/JHB/34/94	320	<40	**2560**	2560	160
A/Thess/1/95	640	160	640	**2560**	40
A/Wuhan/359/95	160	<40	40	80	**640**

Numbers represent reciprocal values of the end point haemagglutination titre. Where a homologous virus and antiserum have been reacted, the end point titre is highlighted in bold and underlined. ND – Not Done.

In order to relate the antigenic changes to the sequence of the HA, the HA1 region was sequenced from each individual GS isolate. It was thought that sequencing this particular region would give the best "picture" of changes in the virus that were occurring due to selection pressures. Amino acid alignments were constructed from the 347 residue HA1 sequence using the GS viruses and a selection of field isolates that had circulated prior to and after the study period. Two prototype virus strains were also included in the alignment, A/Thess/1/95 and A/Wuhan/359/95. At the time of the GS infection, viruses circulating the community had been A/Thess/1/95-like; towards the end of the GS infection the circulating strains had drifted to an A/Wuhan/359/95-like virus. The HA1 sequence of A/Thess/1/95 was used as the consensus to which all sequences were compared (Figure 6.4). Overall, the HA1 region was relatively conserved between all viruses. There were three positions where mutations had occurred that appeared to have some significance. At position 158, the majority of the GS viruses carried a mutation that was not observed in any of the field strains sequenced. GS1, GS1B and GS4 all shared residues with the A/Thess/1/95 prototype but the remaining nine GS viruses all carried either Glu158Gly (1/9) or Glu158Lys (8/9) mutations. It appeared that the appearance of the Glu158Lys mutation in GS3 was then fixed in the virus population over the remaining sequential isolates.

In GS7, a Val196Ala mutation was identified that was then found in the next three sequential isolates. The position of the mutation within HA1 was interesting because it was located within antigenic site B (Figure 6.4). The fixation of this mutation through the next three sequential isolates representing a period of approximately twelve months suggested that the viruses carrying the mutation had acquired a selective advantage resulting in the predominance of this genotype within the viral quasispecies. The sequence alignment also revealed that GS10 had also acquired a change within another area of antigenic importance. The virus contained a His75Asn mutation within antigenic site E (Figure 6.4).

FIGURE 6.4 Amino acid alignment of HA1 from GS sequential virus isolates, field strains and prototype viruses



Influenza A HA1 antigenic sites: A B C D E

HA1 sequences are aligned to the 1995/96 prototype virus A/Thess/1/95.

Viruses that had been isolated on the same day were analysed for sequence homology. GS1A and GS1B were isolated from a nose swab and gargle washings respectively. The sequence of HA1 revealed that the two viruses differed; GS1B was homologous to GS1 in sequence but GS1A carried a Glu158Gly mutation. Similarly, GS4 and GS5 were isolated from gargle washings and a nose swab; these two viruses had heterogeneous sequences. Two amino acid changes were identified between the two viruses; GS4 carried a Ser45Ile mutation and GS5 had a Glu158Lys mutation. The final two samples taken simultaneously were GS7 and GS8; both were gargle washings. The two viruses isolated from the samples shared complete sequence homology of HA1.

When the results from the HA1 sequencing and the HAI assay were compared, a correlation was observed between the two sets of data. The appearance of the Val196Ala mutation in GS7, GS8, GS9 and G10 correlated with a drop in reactivity demonstrated in the HAI assay. It was therefore hypothesised that this was evidence that the mutation has affected the virus in its capacity to either bind with antibody or to the receptors on the erythrocytes used in the experiment. In an attempt to prove this idea a further set of experiments were performed to assess the receptor binding properties of the GS viruses carrying the Val196Ala mutation.

6.4.4 Analysis of the Receptor Binding Properties of GS Viruses

The occurrence of the Val196Ala mutation had been demonstrated by sequencing the HA1 region of the GS viruses (Figure 6.4). The possible consequence of this mutation on the antigenic or receptor binding properties of the viruses had been illustrated from the HAI data. Therefore, in order to ascertain whether the mutation did affect the potential affinity to bind to sialic acid receptors on erythrocytes, a simple receptor binding experiment was performed. This involved titrating GS1 (lacking Val196Ala mutation) and GS7, GS8, GS9 and GS10 to a known dose and then mixing with a range of erythrocytes originating from different species. The binding affinity of the GS viruses to turkey, guinea pig, chick and human erythrocytes were all analysed; the chick erythrocytes produced the most interesting result (Table 6.5). It appeared that GS1 bound with a relatively high affinity compared to the last four viruses. GS7 demonstrated some evidence of

binding however GS8, GS9 and GS10 all completely lacked the ability to agglutinate the chick erythrocytes. The experiment was performed at different temperatures to ensure that such variables were not influencing the final outcome of the test; the results were almost identical at each reaction temperature. This was evidence for the potential effect of Val196Ala in HA1 on virus function.

TABLE 6.5 Affinity of GS haemagglutinin binding to chick erythrocytes

Virus	Incubation Temperature					
	4°C		RT*		37°C	
A/Beij/359/89	8	8	8	8	4-8	8
A/Eng/301/95	2	2	2	2	2	2
A/Wuh/359/95	8	8	8	8	8	8
A/Syd/5/97	4	4	4	4	4	4
A/Eng/729/97	8	8	4	4	8	8
GS1	4	4	4	4	2	2
GS7	+/-	+/-	+/-	+/-	+/-	2
GS8	<2	<2	<2	<2	<2	<2
GS9	<2	<2	<2	<2	<2	<2
GS10	<2	<2	<2	<2	<2	<2
-VE Control	<2	<2	<2	<2	<2	<2

*** RT – room temperature. Numbers represent the haemagglutination titre of virus. +/- represents wells where the HA titre could not be distinguished between an undetectable value (<2) and a HA titre of 2.**

6.4.5 Determination of the Immune Status of GS Serum Samples

A series of serum samples had been taken from GS (GSa to GSh) prior to, and during the persistent influenza infection (Figure 6.1). In order to determine the extent of the humoral immune response raised to the infection, a series of assays were used to measure levels of specific antibody within the samples. Initially, a HAI assay was used. The GS serum samples were titrated against a panel of reference viruses selected as those strains representing the viruses thought to be circulating at the time of, or prior to the GS infection. Results demonstrated an inability of the serum samples to inhibit the agglutination properties of the HA within the assay. This provided evidence that there were very low levels of specific anti-HA antibody present within GS (Table 6.6). The virus strain most antigenically similar to the GS viruses, A/Thess/1/95, should have been the virus most likely to demonstrate some inhibition of agglutination, however the serum samples contained no apparent antibody titre to this virus even after eighteen months of infection.

Another method of detecting anti-influenza antibody activity within the samples was to assess the neutralising capacity of the GS sera. This technique, performed as a microneutralisation test was designed to detect the presence of neutralising antibody within the GS serum samples (262, 308). It was possible that although the HAI assay had not detected any specific anti-HA antibody, there could be some neutralising activity present. The samples were titrated against a panel of reference viruses and the resulting serum-virus complex was tested for its ability to infect cell monolayers, which was subsequently quantified by a modified EIA (Table 6.7). The results from the test showed that each serum sample had failed to raise any significant response to any of the viruses in the reference panel. The titres produced from the assay were all low enough to provide evidence that neutralising antibody within the GS sera failed to inhibit the infection of virus particles in the EIA. GSg showed some evidence of neutralising activity against A/Thess/1/95 and A/Shan/9/93 suggesting that at the time of sampling, the neutralising antibody level within GS had been raised (Table 6.7).

TABLE 6.6 Haemagglutination inhibition of GS serum samples against a panel of reference influenza strains

GS Serum	Date	A/Taiwan/1/86	A/Texas/36/91	A/Thess/1/95	A/JHB/34/94	A/Shan/9/93	B/Pan/45/90
Gsa	18/05/94	<5*	<5	<5	<5	<5	<5
GSb	07/02/95	<5	<5	<5	<5	<5	<5
GSc	26/07/95	<5	<5	<5	<5	<5	<5
GSd	03/08/95	<5	<5	<5	<5	<5	<5
Gse	10/08/95	<5	<5	<5	<5	<5	<5
GSf	20/09/95	<5	<5	<5	<5	<5	<5
GSg	12/07/96	<5	<5	<5	<5	<5	<5
GSh	26/09/96	<5	<5	<5	<5	<5	<5
+VE		1280	640	320	1280	1280	160
-VE		<5	<5	<5	<5	<5	<5

*** Numbers represent the end-point titre of agglutination. <5 represents a titre that could not be detected.**

TABLE 6.7 Microneutralisation of GS serum samples

GS Serum	Date	A/Taiwan1/86	A/Texas/36/91	A/Thess/1/95	A/JHB/34/94	A/Shan/9/93	B/Pan/45/90
Gsa	18/05/94	40	<5	<5	40	40	<5
GSb	07/02/95	40	20	40	<5	20	<5
GSc	26/07/95	20	40	80	20	20	<5
GSd	03/08/95	40	80	80	20	20	<5
Gse	10/08/95	20	40	80	20	40	<5
GSf	20/09/95	20	20	40	20	20	<5
GSg	12/07/96	80	80	160	80	160	<5
GSh	26/09/96	40	<5	<5	40	40	<5
+VE		5120	2560	>5120	>5120	2560	640
-VE		<5	<5	<5	<5	<5	<5

Positive and negative controls consisted of ferret antiserum homologous to the reference viruses and a serum taken from a negative ferret respectively.

6.4.6 Cloning of GS1 and GS10 to Determine the Quasispecies

To investigate the population dynamics of the GS viruses, a series of cloning experiments were performed. The HA1 of GS1 and GS10 was amplified and cloned. Clones were produced for both GS1 and GS10 and then the HA1 gene contained within each clone was amplified and sequenced. Nucleotide and amino acid alignments were constructed and analysed for the presence of residue changes. In total, seventeen clones were analysed, eight GS1 and nine GS10 clones. The data demonstrated that there was greater heterogeneity within the HA1 sequence of the GS1 clones compared to GS10 (Table 6.8; Appendix 25 and 26). Mutations were broken down into different categories; transition, transversion, synonymous and non-synonymous. The occurrence of mutations within antigenically important regions i.e. antigenic sites was also noted. In general, mutations of all categories occurred more frequently within the GS1 clones that GS10. Within the antigenic regions, there was a predominance of non-synonymous (coding) changes over synonymous. When amino acid changes were calculated as a percentage of the total site available for change (279 non-antigenic and 68 antigenic residues) this was confirmed. Within the GS1 and GS10 clones, non-synonymous mutations occurred in 11.8% and 4.4% respectively of the residues available compared to 9% and 2.9% of non-antigenic residues for GS1 and GS10 respectively.

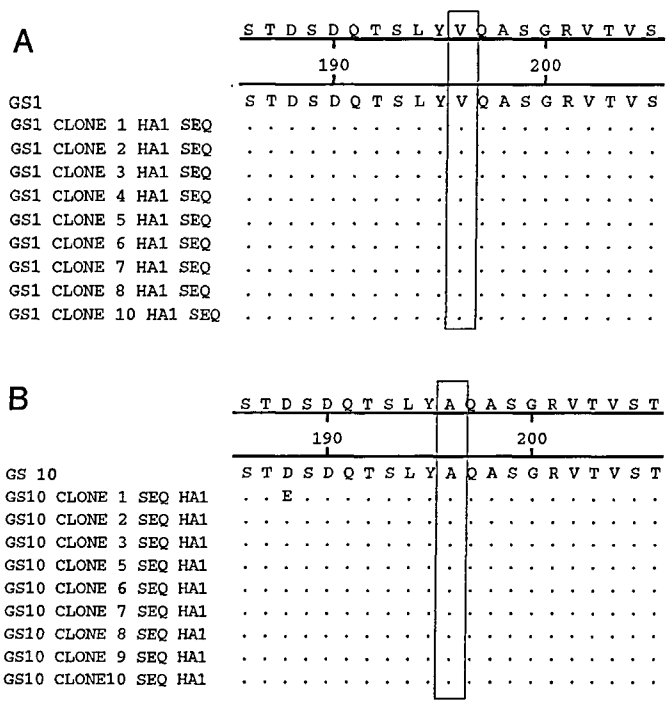
From the sequence alignments produced for each set of clones, it was possible to confirm the presence of mutations initially found during the original sequencing of the GS viruses. The mutations of interest were His75Asn (GS10) and Val196Ala (GS7, GS8, GS9 and GS10), both mutations were located within antigenic sites of the HA1 molecule. Sequence analysis of the GS1 clones revealed that none of the clones contained either mutation. All the GS10 clones were found to contain both mutations (Figure 6.5; Appendix 25 and 26). This suggested that the Val196Ala mutation was fixed within the viral population. In a series of additional experiments performed by J. Ellis (Respiratory Virus Unit, CPHL), a limiting dilution of GS10 was made and PCR amplification and sequencing performed on the resulting material. Results demonstrated that at the end point of PCR detection, the viral species found contained the Val196Ala mutation illustrating the dominance of this sequence.

TABLE 6.8 Sequence diversity of HA1 within GS1 and GS10 clones

		Number of Changes						
Clone	**N**	**Total**	**Ts**	**Tv**	**Syn**		**N-Syn**	
					Ant	**Non-Ant**	**Ant**	**Non-Ant**
GS1	8	49	32	17	2	13	8	26
GS10	9	13	12	1	0	2	3	8
Total	**17**	**62**	**44**	**18**	**2**	**15**	**11**	**34**

Sequence changes within each set of clones were compared to the sequence of the original virus isolate of GS1 and GS10. N – number of clones; Ts – transistion; Tv – transversion; Syn – synonymous; N-Syn – non-synonymous; Ant – antigenic region; Non-Ant – non-antigenic region.

FIGURE 6.5 Amino acid sequence of the HA1 region of (A) GS1 and (B) GS10 clones



Clones were aligned to the sequence of the original GS1 or GS10 virus isolate. Only residues 186-206 of the HA1 region are displayed. Amino acid residue 196 is boxed to demonstrate the heterogeneity between, and the homology within, the two sets of clones at this position. See Appendix 25 and 26 for complete HA1 alignments.

6.4.7 Evolution of the M1, M2 and HA1 Originating from the GS Viruses

The rate of nucleotide and amino acid changes for the GS viruses and field strains isolated over the study period was calculated for the M1, M2 and HA1 proteins Tables 6.9 to 6.11). The rate of change of M1 was calculated for the GS and field strains using the methods described in Chapter 5 (page 161). The results showed that the field strains had undergone much greater evolutionary change in respect to both nucleotide and amino acid over the study period compared to the GS viruses. It seemed that there was an unusually high rate of change within both the GS and field viruses (Table 6.9). The number of GS viruses available for this analysis was restricted to five isolates. The relatively small number of samples within both data sets may have influenced the overall values for rate of change calculated for M1.

TABLE 6.9 Rate of change of M1 (amino acid and nucleotide) for GS and field isolates

Virus	N	Total nt/site/year	Total aa/site/year
GS1 – GS9*	5	8.8×10^{-3}	3.9×10^{-3}
Field Isolates**	11	21.6×10^{-3}	14.1×10^{-3}

*** The GS viruses used for M1 analysis were GS1, GS1B, GS3, GS5 and GS9. ** Fieid isolates were A/Thess/1/95-like in antigenicity. N – number of viruses.**

The rate of change of M2 from the GS and field strains was calculated (Table 6.10). Values were much lower than those calculated for M1.

TABLE 6.10 Rate of change of M2 (amino acid and nucleotide) for GS and field isolates

Virus	N	Total nt/site/year	Total aa/site/year
GS1 – GS10	12	0.09×10^{-3}	0.2×10^{-3}
Field Isolates*	12	0.1×10^{-3}	0.08×10^{-3}

*** Field isolates were A/Thess/1/95-like in antigenicity. N - number of viruses.**

The rate of change of the HA1 region of the GS and field strains was calculated (Table 6.11). A selection of data from previous studies was included to enable further comparisons (59, 96). The rate of change of the field strains from all three studies was comparable. When the values for the GS viruses were analysed it was apparent that there were elevated levels of change compared to the field strains. This was especially noticeable for the amino acids, which were evolving at a rate of 13.15×10^{-3} residues per site per year (Table 6.11). This represented an increase in excess of two-fold between the rate of amino acid change within the GS viruses and the field strains from the present study.

TABLE 6.11 Rate of change of HA1 (amino acid and nucleotide) for GS and field isolates

Sample	Period of Circulation	N	Total nt/site	Total aa/site	Reference
H3N2	1968-1994	183	3.65×10^{-3}	5.83×10^{-3}	(59)
H3N2	1984-1996	254	5.7×10^{-3}	9.7×10^{-3}	(96)
Field Isolates*	1995-1996	20	4.45×10^{-3}	4.42×10^{-3}	Present study
GS1 – GS10	1995-1997	12	6.56×10^{-3}	13.15×10^{-3}	Present study

*** Field isolates were A/Thess/1/95-like in antigenicity. N - number of viruses.**

6.5 Discussion

The above case study presented an ideal opportunity to study the progression of a persistent influenza infection. As previously stated, to our knowledge this represented the longest documented period of persistence, twelve influenza A viruses were isolated and characterised over an eighteen-month period.

6.5.1 Phenotypic Analysis of GS Viruses

The phenotypic characterisation of the GS viruses by susceptibility EIA and plaque reduction assay revealed that the viruses isolated seemed to be composed of different drug susceptibilities (Figure 6.2 and Table 6.2). The first two isolates, GS1 and GS1A were amantadine-sensitive by both methods. However, the period of amantadine treatment coincided with a reduction in susceptibility to the drug. Amantadine treatment was initiated prior to the time that GS1A and GS1B were isolated but phenotypic analysis seemed to suggest that although isolated on the same day, these two viruses differed in their susceptibility to amantadine being sensitive and resistant, respectively. The available data showed that the presence of drug treatment had resulted in an overall reduction in amantadine susceptibility. The generation of amantadine-resistant populations as a direct result of amantadine treatment has been previously documented. The rate at which resistant viruses were isolated in the present study was extremely quick. Clinical details showed that amantadine had been administered on 29/06/95 lasting for ten days. GS1A and GS1B had been isolated from samples taken from the patient on 30/06/95. Therefore, it had only taken approximately one day of amantadine treatment to reduce the overall susceptibility of the virus population to the drug, apparently rendering the viruses resistant. This finding illustrated the major problem in using amantadine or rimantadine in the clinical treatment of influenza A infections. The generation of a resistant virus population was almost immediate and it was maintained for a prolonged period even without the selective pressure of drug.

There were several GS isolates (GS3, GS5 and GS8) that had negative values for reduction in PFU/ml (Table 6.2). These results suggested that virus yield had increased in the presence 1.0 µg/ml amantadine. The most striking result was that

of GS8, which demonstrated a relative increase in PFU/ml of over thirty percent in the presence of amantadine. The reasons behind this phenomenon were not clear; there have been no similar reports to the findings made here. However, results from the amantadine susceptibility screening of UK influenza A isolates demonstrated similar findings (Table 3.7, page 75). From these findings it was hypothesised that the dissociation of the M1/RNP complex could occur at higher pH to compensate for the biochemical effects of amantadine. This could also have explained the results found in the GS viruses. It seemed that in chronological order, the three viruses (GS3, GS5 and GS8) displayed increasing negative values (Table 6.2). This may have indicated that over the study period, variants carrying M1 species with the ability to compensate for the antiviral effect of amantadine accumulated producing the above results. The implications of some of these findings were that the amantadine-resistant GS viruses had heterogeneous growth properties. The mean plaque sizes of each virus grown in the presence and absence of drug were measured (Table 6.2). This highlighted an apparent reduction in the size of plaques from highly sensitive viruses suggesting that the growth potential of the virus in addition to the PFU/ml was reduced in the presence of amantadine. Plaques isolated from viruses with negative PFU/ml values, grown in the presence of amantadine, seemed to be similar in size to those plaques grown without drug. This suggested that these resistant variants were able to maintain viral growth in the presence of drug possibly demonstrating adaptation to the conditions.

6.5.2 Genetic Characterisation of M2

Sequence analysis of the M2 protein originating from the GS virus isolates demonstrated the emergence of an amantadine-resistant genotype. When these data were analysed in parallel with clinical details, there was a correlation between the course of amantadine treatment administered after the initial diagnosis of influenza and the appearance of a population of resistant viruses. Therefore, it was apparent that the treatment with amantadine had been sufficient to provide the pressure required to select resistant variants within the host. The emergence of the resistant virus population was rapid; GS1B, genetically characterised as resistant, was isolated within a few days of the initiation of drug treatment. These findings supported results from other studies that have reported the rapid

emergence of drug-resistance in patients undergoing amantadine and rimantadine treatment (147, 193).

The nature of the mutations occurring within the M2 transmembrane domain was consistent with previous reports defining the changes required to confer amantadine-resistance to the virus (118, 142). The most frequent mutation was Ser31Asn (7/12), which provided further evidence for the common nature of this mutation within amantadine-resistant human influenza A viruses and supported results from Chapter 4 and previous studies (90, 144, 147, 166, 417). However, sequencing M2 of the remaining sequential GS isolates illustrated the heterogeneity of the virus population in respect to amantadine-resistance. The Ser31Asn mutation in GS1B, the first resistant virus isolated, was not fixed throughout the remaining viruses. Other mutations included Val27Ala (3/12) and Ala30Thr (1/12), similarly these mutations had been previously described in human viruses resistant to amantadine (147, 166, 193, 238). In addition, there was no temporary fixation of mutations, i.e. specific mutations emerged, were superseded by another mutation and then were seen to re-emerge as the dominant genotype at a later stage of the infection; there seemed to be random selection of the resistance genotypes.

Following the emergence of a resistant viral population within GS, it was interesting to note that this genotype was maintained in the absence of any selective pressure from the drug. Eighteen months after drug treatment, GS was still shedding resistant virus. The implication of this finding was that once the resistant genotypes had become dominant within the viral quasispecies, they were able to survive and possibly outcompete the wild-type sensitive viruses. It has been previously documented in certain animal models that amantadine-resistant viruses are unaltered in virulence and growth characteristics compared to sensitive viruses (22, 350); the results from this study support these findings. Once established, the resistant variants remained dominant demonstrating that in the absence of the selective pressure they remained at a selective advantage over the sensitive genotypes.

In some isolates, mixtures of sensitive and resistant genotypes were discovered e.g. GS2, GS3 and GS6 (Table 6.3). It was hypothesised that because GS2 and GS3 were isolated during and immediately after the amantadine treatment

respectively, they would contain a higher proportion of sensitive genotypes. When the correlating plaque reduction assay and drug susceptibility-EIA data were analysed (Figure 6.2), it appeared that GS2 demonstrated an increased susceptibility to amantadine compared to other resistant viruses subsequently isolated. This finding supported the sequencing data, it appeared that in the short period between initiation of drug treatment and isolation of GS2, there had been insufficient time for the resistant population to become completely dominant and sensitive genotypes could still be readily detected. GS3 also contained a mixed population of resistant and sensitive genotypes but the overall drug susceptibility of the virus by plaque reduction assay and the susceptibility-EIA was low. Although mixed genotypes were found in GS3, overall there might have been a higher proportion of resistant variants that would have reduced the susceptibility as detected by plaque reduction and EIA. The mixed populations of genotypes found in this study are supported by previous work that determined that once drug-resistant virus has emerged from a subject, the genotype of subsequent isolates remains resistant or comprises a mix of resistant and sensitive viruses even in the absence of drug treatment (22).

In order to determine the population dynamics of heterogeneous populations of sensitive and resistant variants, it would be interesting to perform a competition study. After co-infecting cell monolayers with known amantadine-resistant and sensitive viruses the progression of infection could be monitored. At time periods throughout the infection, samples could be taken to estimate the proportion of genotypes present within the culture. This type of experiment would provide data concerning the competition between resistant and sensitive genotypes under "normal" growing conditions. To simulate the present case study, amantadine could be introduced to the cell system and the effect of the selective pressure on the population dynamics monitored. The data would provide an indication of the delicate balance between resistant and sensitive genotypes in a mixed population of virus. The addition of amantadine would also reveal how that balance would change with the selective pressure introduced, whether the resistant genotype would dominate or mixed populations could still be readily detected.

The heterogeneity of the M2 transmembrane domain from the GS isolates was evidence for the presence of quasispecies within the host. This supported previous studies that had reported similar findings (193, 304). The genetic variability

observed was probably a reflection of the fluctuations within the viral quasispecies, i.e. there was continuous competition between variants. The results also demonstrated the heterogeneity within the resistant strains. Even though the pressure from amantadine treatment had selected a resistant population, there was still significant heterogeneity of variants carrying different resistance traits. This was especially evident within GS3 where a mixture of resistant variants was found carrying Ala30Thr and Ser31Asn mutations. This demonstrated that at any one time there were mixed populations of resistant variants within the viral quasispecies.

An interesting observation was made concerning viruses that were isolated from different anatomical sites at the same time. On three separate occasions viruses were isolated on the same day. GS1A and GS1B were isolated from a nose swab and gargle and were characterised as amantadine-sensitive and –resistant, respectively. GS4 and GS5 were isolated from a gargle and nose swab and were found to carry different mutations within the M2 transmembrane domain, Val27Ala and Ser31Asn respectively. The final pair of viruses, GS7 and GS8 were isolated from separate gargle samples taken on the same day but these two viruses shared complete sequence homology of the M2 transmembrane domain. It appeared that viruses isolated from different sample sites were genotypically different in respect to M2. It was difficult to explain these findings, but it was hypothesised that the most logical reason was the presence of quasispecies within the host. Sampling and PCR amplification had simply detected different variants. Alternatively, it was postulated that variants with different drug genotypes might have been restricted to different sample sites. It has been previously reported that amantadine-resistant viruses with different mutations within the M2 transmembrane domain potentially have different growth rates (118). It is possible that resistant viruses carrying different mutations may also demonstrate some form of cell tropism as a result of varying growth and replication rates. Although there has been no reported evidence for this theory, the idea of compartmentalisation of HIV-1 virus has been documented (1, 15, 293, 367). It has been shown that resistance to zidovudene (ZDV) exhibited by HIV-1 can be heterogeneous between different organ or tissue systems due to the increased replication rate of the virus that allows the generation of a greater number of variants (15). Similarly, it has been reported that the distribution of HIV-1 variants carrying different point mutations within certain organs or tissue types can vary

(15). It would be interesting to perform an experiment to assess the potential compartmentalisation of different amantadine-resistant genotypes. Theoretically, this could be achieved by using an animal model. The animal would be infected with a known population of mixed sensitive and resistant genotypes and after a given period, tissue samples from various sites could be isolated and virus extracted to assess the genotype of the variants. This type of experiment could provide evidence for this phenomenon occurring in influenza. Alternatively, if disproved, then it would appear that the presence of quasispecies and random amplification and sequencing of different variants would explain the above results.

6.5.3 Evolution of GS and Field Strain M1 and M2

The rate of change of M1 and M2 isolated from the GS viruses, and a selection of field strains that had been circulating over the study period was calculated. Values gained for M1 appeared to be high for both the GS and field strains compared to previous findings from the present study (Chapter 5, page 165) and other published results (175). This might have been the result of a smaller number of viruses analysed. The rate of change of M1 in the GS viruses was lower when compared to the rate in the field isolates. It was possible that this was supporting evidence for the severely suppressed immune system in GS. M1 has been shown to be the target of cytotoxic T lymphocytes in the immune response, therefore if the immune system in GS was compromised, the immune pressure on M1 would not be as significant as M1 from the field isolates (116, 176, 296). The rate of change of M2 from both GS and field isolates was similar but lower than previous results from this study and others (175). It was unclear to why these values were lower but it was possible again that the small number of viruses analysed might have biased the results.

6.5.4 Antigenic Characterisation of GS Viruses

The antigenic profile of each GS virus was assessed. Results obtained strongly suggested that antigenic drift had occurred from GS1 to GS10. Under normal conditions, the pressure from the host immune system exerted upon the surface glycoproteins of the influenza virus drives changes in the form of nucleic acid and

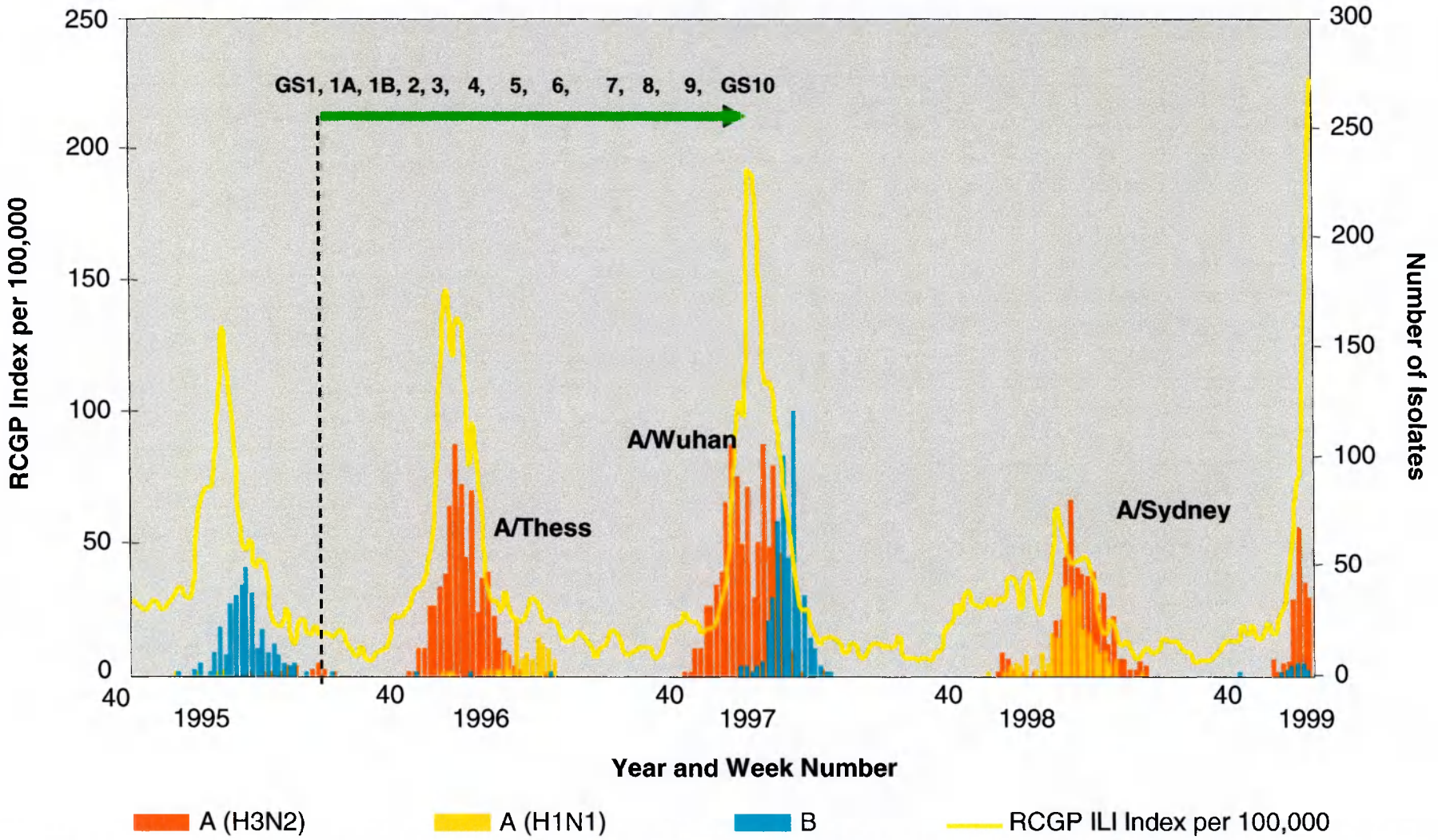
amino acid mutations (59). This gradual process results in the drift of the virus that can be detected by cross-reacting with antisera raised against specific antigenic types of virus (performed by HAI). Typically, a fall in titre of approximately four-fold indicates antigenic drift has occurred. HAI studies on the GS viruses had demonstrated the GS10 had an eight-fold reduction in end-point titre with A/Thess/1/95 when compared to GS1 (Table 6.4). Therefore, it appeared that over the eighteen-month study period, GS10 had significantly changed in its antigenicity when compared to the first virus isolated (GS1). Analysis of the GS serum samples had provided evidence that there had been very little humoral immune response to the influenza infection. Specific and neutralising antibody could only be detected at very low levels in certain samples and was below the threshold for detection in others. Analysis of the clinical details of GS revealed that throughout the study period, several courses of immunoglobulin (IgG) had been administered to GS, presumably in an attempt to boost the immune system. It was hypothesised that this could have resulted in certain serum samples having slightly elevated antibody titres. For example, analysis of the neutralising potential of the GS serum samples revealed that GSg had elevated antibody titres compared to the other samples (Table 6.6). When the following serum sample (GSh) was tested, the titre of antibody had fallen to a level that was barely detectable with the microneutralisation assay. It was possible that the GSg sample had been taken immediately after an IgG booster, which might have influenced the results. It was concluded that the data from the neutralisation and HAI testing of serum provided evidence that within GS there was minimal selective pressure from the humoral immune system.

Therefore, the immunological evidence had shown that antigenic drift had occurred from the isolation of GS1 to GS10, a period of eighteen months. There was strong evidence that the immune system of GS was severely depleted i.e. CD4 undetectable, and that a minimal response had been raised against the influenza H3N2 infection. Antigenic drift had been observed in a system that lacked the driving force of immune pressure. This was an extremely interesting observation. The only other reported case similar to this involved persistent infection of a H3N2 virus in an immunocompromised host (244). Similarly, the virus was observed to drift, in this case from an A/Wuhan/395/95-like to A/Syd/5/97-like strain over the period of infection (twelve months). It was postulated that the drift was driven by humoral immunity. However, it cannot be

excluded in this case that the patient was reinfected with a virus of differing antigenicity. The pattern of viral drift mirrored changes that had occurred within the community during the period of infection. In addition, there was a transition phase where an accurate antigenic profile of the virus had not been possible. Therefore, an alternative explanation for these findings could be that the host had acquired a nosocomial influenza infection from the community when the antigenicity of the circulating virus had drifted to the novel form. This would have resulted in the observed results, implying that the virus had drifted within the host.

This was a serious consideration for the GS viruses, that the persistent infection might have actually been composed of several separate infections. To prove that the drift within GS was not caused by the acquisition of a novel infection, a chart illustrating the antigenic subtypes of community-acquired influenza during the 1995-1999 influenza seasons was constructed (Figure 6.6). The HAI of the GS viruses had shown that they were A/Thess/1/95-like in profile. Although there was evidence to show that GS10 had drifted, it had remained more A/Thess/1/95-like than A/Wuhan/359/97-like (Table 6.4). When this data was compared with Figure 6.12, it could be seen that GS10 had been isolated at a time when the virus circulating the community was A/Wuhan/359/97-like. However, it had been shown that GS10 was more antigenically related to A/Thess/1/95, evidence that GS10 was not the result of reinfection, but was the end result of a strain that had drifted from GS1. This was further ruled out by the presence of the drug-resistant phenotype and genotype in GS10. This characteristic is very rare in natural isolates and was supported by the drug susceptibility screening of the 1995/96 influenza season, there were no naturally occurring amantadine-resistant viruses detected within the UK (Chapter 3, page 72).

FIGURE 6.6 Shedding of influenza virus from GS compared with predominant circulating viruses from 1995 to 1999



Bars represent the level of influenza activity in the UK population based upon virus-isolation rates. The RCGP index highlights the close association of consultations for influenza-like-illness and the isolation of virus.

Sequencing the HA1 region of the GS viruses and other field and prototype strains had revealed amino acid changes at certain positions between the A/Thess/1/95-like and A/Wuhan/359/95-like strains. Previous reports have defined antigenic drift variants to differ by at least four amino acid changes within two antigenic regions (59). The A/Wuhan/359/95-like viruses contained two to three amino acid changes located in antigenic sites when compared to A/Thess/1/95. Therefore, although the sequence changes between these two prototype viruses did not fit with the definition of drift variants, it had demonstrated that over the time that the two viruses had been isolated, changes had occurred resulting in the formation of the drift variant (A/Wuhan/359/95). Compared to the sequence of A/Thess/1/95, GS10 contained two amino acid mutations within antigenic regions demonstrating the apparent drift that had occurred within the GS viruses culminating with this final isolate. Therefore, the molecular data appeared to confirm the findings from the HAI testing of the GS viruses.

Within the GS viruses, HA1 sequencing had revealed a mutation (His75Asn) in the last virus isolated (Figure 6.4). His75Asn was located within antigenic site E but it was difficult to postulate the significance of this mutation because there was no evidence that it had been fixed within the virus population. To investigate this further, viruses isolated after GS10 would have provided the evidence required to assess the importance of the mutation. The two amino acids differed in their structures quite considerably, His is positively charged and Asn is uncharged (314). The size of His and Asn was similar, 155 and 132 Daltons respectively but the side groups attached to each residue differed, His is associated with a basic side group and Asn with an amide (314). It was postulated that this His75Asn mutation, occurring within an antigenic domain of HA1 would have the potential to cause change within the protein. There were no viruses isolated after GS10 and therefore it could not be determined whether the mutation had been fixed. If there had been evidence to show fixation of the mutation within the virus population, it may be concluded that the difference between the two amino acid residues might have been sufficient to cause changes within the receptor-binding site to affect virus function.

The HA1 sequencing had revealed the presence of another mutation that occurred in a region of antigenic importance (Figure 6.4). Sequencing the HA1 region of the GS viruses revealed a fixed mutation (Val196Ala) in the last four viruses isolated.

This mutation was known to be located within the site of a series of conserved residues that constituted an antigenic domain (393). Due to the location of this mutation it was hypothesised that some function of the virus, e.g. affinity for host cell receptors or antibodies, might have been affected. Both Val and Ala are non-polar, hydrophobic molecules with aliphatic side chains and of 117 and 89 Daltons in size respectively (314). Therefore, the two residues are very similar in character and it was concluded that the Val196Ala mutation would not have caused major structural changes to the HA1 region.

The receptor binding properties of the viruses with and without Val196Ala were assessed. It seemed that the viruses carrying the mutation had reduced affinity to bind to chick erythrocytes. It was postulated that this provided evidence that the mutation had affected the function of the virus in respect to its binding properties. Although it had been concluded that the similar natures of the two amino acids involved would not cause significant structural changes within HA1, it appeared that the mutation had been sufficient to affect the protein function. The fixation of Val196Ala from GS7 to Gs10 implied that the presence of the mutation had conferred a selective advantage. It was postulated that if the mutation had been detrimental, Val196Ala variants would have been lost in the viral quasispecies because they represented an evolutionary "dead-end". If this had happened, the mutation may have been evident within GS7 but would not have been detected in the next three sequential viruses. To determine whether the mutation was actually fixed, and not an artefact of the amplification process randomly selected and sequenced, data from the HA1 cloning was analysed. Each clone of GS1 was found to have the wild type Val residue at position 196 and all GS10 clones carried the mutation (Figure 6.5). The receptor binding study had also shown that GS7 differed slightly from the other three viruses carrying the Val196Ala mutation in its ability to agglutinate chick erythrocytes. It was postulated that GS7 might have represented a transition state where there was a mixture of wild-type and mutant variants within the viral quasispecies. If the mutants were at a selective advantage over the wild-type then the proportion of variants carrying the Val196Ala mutation would have increased until GS8 where the majority would have carried the mutation. This would have accounted for GS7 retaining the ability to agglutinate despite carrying the mutation.

The selective advantage that the Val196Ala mutation conferred to GS7, GS8, GS9 and GS10 is difficult to explain. It had been demonstrated that the potential for receptor binding had been reduced but it was not clear how this change in function could carry a selective advantage to the virus. The alteration in receptor binding capacity of the last four GS viruses might have been related to the adaptation of the viruses to the localised environment within GS. The mutations may have increased the specificity of the HA molecule to host cell receptors, allowing the virus to increase the rate at which attachment and penetration into host cells occurred. This mutation may also have enabled the virus to persistently infect the patient. In certain influenza viruses it has been postulated that mutations or variation within certain genes allow the virus to maintain a persistent infection (208, 234). Therefore, it was possible that this mutation within the HA1 region of the later GS viruses, had in some way permitted the virus to maintain the infection.

Alternatively, it is possible that a change within this region of antigenic importance could have affected some aspect of antibody binding to the protein. Although it had been shown that antibody levels were very low and clinical details had revealed a very low CD4 count within GS, quantitative analysis of other components of the immune system, e.g. T cells, killer cells, neutrophils etc. was not possible. It was postulated that a mutation within antigenic site D might have enabled the virus to escape detection from one of the above immune components. In support of this theory is evidence that demonstrates the presence of possible epitopes for T lymphocytes within the HA1 region. Several distinct overlapping epitopes have been mapped to HA1 residues 56-76, 71-91, 81-97, 177-199, 186-205 and 206-227 (18, 42). Within the 177-199 region, changes of single amino acid residues at positions 198 and 199 have been shown to cause differences in T cell recognition (19). Therefore, these data provide a possible explanation for the fixation of Val196Ala. It was possible that the mutation enabled the virus to escape recognition by T lymphocytes. Within GS, T cells may have provided the only form of immune response. Therefore, the acquisition of this mutation would have provided a distinct selective advantage over other variants within the virus population. This might have explained why this particular mutation was fixed in subsequent viruses over a period of at least twelve months. Interestingly, the His75Asn mutation described in GS10 was also located within a T lymphocyte epitope (residues 56-76). Although it had been postulated that this mutation was relatively severe in respect to differences between the His and Asn residues and

therefore may have affected virus function, if it enabled escape from T lymphocyte detection it would have been advantageous to the virus. This might have resulted in the fixation of this mutation in the virus population and would have been detected in subsequent isolates after GS10 if they had been available for analysis.

The cloning of the first and last GS viruses confirmed the absence and presence of Val196Ala in antigenic site D of GS and GS10 respectively. Further analysis of the cloning work enabled conclusions to be drawn concerning other areas of the work. It was hypothesised that over the period of the persistent infection, changes within the overall viral quasispecies would occur. It had been demonstrated that there was minimal immune response to the virus and therefore there was low selective pressure slowing the progression of infection. Without this restriction, the GS viruses had the opportunity to replicate at elevated levels, speeding up the overall rate of change of each virus over the persistent infection. Evidence for this was generated when the rate of change of HA1 for the GS viruses and field strains representing A/Thess/1/95-like viruses circulating the community at the time of infection was calculated (Table 6.11). It was apparent that the rate of change of nucleotides and amino acids was much higher within the GS viruses compared to field strains and other published rates (59, 96). It was concluded that this was evidence to suggest that the elevated rates of change in the GS viruses were as a consequence of the depleted immune system of the host. From this, it was logical to postulate that increased replication rates would have given rise to the generation of greater numbers of variants within the viral quasispecies, especially in the later GS viruses. However, the cloning work demonstrated that GS1 contained more sequence variation than GS10 in respect to HA1 homology between clones (Table 6.8). There were several possibilities to explain the results presented here.

The unrestricted progress of the persistent infection might have led to a situation where competitive selection occurred between variants. This had previously been observed within other virus populations (290). The high rates of replication and competition within the viral quasispecies might have led to the emergence of a variant with increased fitness and demonstrating optimal adaptation to the environment. When sampled, this virus population would appear to be less heterogeneous in nature due to the selection of highly adapted variants. Therefore, it seemed that GS10 might have represented a virus population with

increased fitness when compared to GS1. There was strong evidence to show that GS10 had a modified antigenic profile compared to GS1. HAI data demonstrated that the GS10 virus had an end point titre eight-fold lower than GS1 when reacted with the GS1 antiserum. Additionally, when the GS10 antiserum was cross-reacted with the GS viruses, poor titres were observed, supporting the idea that GS10 was significantly different to the other GS viruses. Further evidence to support this theory came indirectly from HAI testing of the GS viruses. To create the ferret antisera required for the test, two preparations of virus (GS1 and GS10) were prepared and inoculated intranasally into separate ferrets. Both viruses had been prepared to ensure that the inoculums contained approximately the same dose of infectious particles. Upon harvesting the sera it was found that the GS1 virus had invoked a good response in the ferret. When the GS10 antiserum was tested there was no antibody detected by HAI. A modified preparation of virus, containing approximately sixty times the dose of GS10 was inoculated into another ferret and the resulting serum harvested was shown to have higher levels of antibody than the first GS10 serum produced, but compared to GS1 the response to the virus had been poor. It was postulated that this could have been evidence of the adaptation of GS1 and GS10 to the environment within GS. It appeared that GS1 was able to replicate efficiently within the ferret producing a good immune response as shown by the antibody titre within the respective antiserum. The evidence suggested that GS10 was highly adapted to the selective environment within GS to such an extent that when presented with novel conditions, the virus failed to thrive and could not raise a strong immune response within the ferret. This form of extreme fitness differences between hosts has been reported for other viruses but there are no available data for influenza to compare the above findings with (263).

A series of future experiments were designed to study the relative fitness of GS1 and GS10. It was proposed that simple growth curves of the virus in cell culture could be obtained initially to demonstrate any differences in growth rate between the viruses. Conditions for replication, e.g. temperature and cell line could be altered to see if the either virus was more efficient at replicating under certain growth conditions. In another experiment, it was proposed that GS1 and GS10 would be reinoculated into ferrets. The condition of each animal would be closely monitored and regular samples taken for analysis would provide evidence of the relative replication rates of each virus within the ferret. It was hypothesised the

results would show that GS1 had the potential to initiate a strong infection and GS10 would struggle as demonstrated by the symptoms presented by, and the overall condition, of each respective animals. However, the result might be misinterpreted as a direct effect of the mutations conferring amantadine-resistance within GS10. The failure of GS10 to cause disease within the ferret might be construed as a result of the reduced virulence caused by amantadine-resistance rather that the hypothesis that GS10 was highly adapted to its host, GS. Therefore, it would be interesting to infect an additional ferret with GS1B. This virus had been isolated early in the GS infection but had the same resistance mutation as GS10 (Table 6.3). Previous evidence has documented the ability of amantadine-resistant viruses to infect ferrets and cause typical influenza (350). Therefore, if GS1B caused infection and disease similar to GS1, this would provide strong evidence that the lack of virulence shown by GS10 was as a result of the extreme fitness difference between GS1 and GS10.

In order to isolate the effect of the amino acid mutations present within the HA1 region of GS10 (His75Asn and Val196Ala), a simple reverse genetics procedure could be employed to create a chimeric virus. The entire HA gene of GS10 could be transfected with the remaining gene segments of GS1 Into a mammalian cell line according to methods already published (105, 358). The resulting virus rescued from culture could then be either inoculated into ferrets or tissue culture to compare the relative fitness to the original GS1 and GS10 virus. These experiments would provide evidence for the HA1 mutations conferring a fitness advantage to GS7, GS8, GS9 and GS10.

One major factor that had to be considered in the molecular population analysis of GS1 and GS10 was the effect of *Taq* polymerase (*Taq*) on the results. It has been shown that *Taq* can cause substitution errors due to the lack of proof-reading mechanisms within the molecule (338). It has been estimated that the error rate for *Taq* can range from 0.2 to 2.0 x 10^{-4} errors per base pair per cycle (227, 312). In a cloning study of vesicular stomatitis virus (VSV), Bracho *et al.* (1998) demonstrated that during the molecular cloning of a PCR product and sequencing of recombinant plasmids, the *Taq* enzyme used in the PCR amplification introduced errors at a rate of 0.27 x 10^{-4} misincorporations per base pair per cycle in addition to mutations present In the natural virus population (36). Therefore, it can be concluded that if the nucleotide substitution rate of a virus was similar or

lower than the misincorporation rate of the DNA polymerase used in PCR reactions, the genetic analysis of the population variables could be affected by such errors. In addition to errors originating from the polymerase molecule, errors produced during the reverse transcription of the virus can occur at a frequency of approximately 1 x 10^{-4} (333). Combined with *Taq* errors, it has been estimated that during a 30 cycle PCR of a 300 base pair product, approximately 0.2 to 2 spontaneous artefactual substitutions will occur (333).

A potential approach to reducing the rate of artefactual substitutions during molecular population studies is to use an alternative polymerase molecule with a lower error rate that *Taq*. One such thermostable enzyme, *Pyrococcus furiosus* polymerase (*Pfu*) has been reported to reduce the error rate by up to 90 percent, significantly reducing the variability of clones and the number of mutations including both synonymous and non-synonymous changes (227). To enable a direct comparison of the fidelity between *Taq* and *Pfu*, the error rate of *Pfu* has been estimated to be between 1.3 x 10^{-6} and 1.6 x 10^{-6} errors per base pair per cycle (50, 227). Although artefactual substitutions occurring during PCR are reduced using a high fidelity polymerase enzyme, errors arising during the reverse transcription stage still continue to occur at a frequency of approximately 1 x 10^{-4} (333). Therefore, by using a polymerase molecule with higher fidelity such as *Pfu*, the number of mutations that could be attributed to polymerase errors would theoretically be reduced.

Unfortunately, a polymerase molecule with a higher fidelity than *Taq* was not used in the present cloning study. However, *Taq* has been commonly used in molecular biology, and many published studies have utilised this enzyme (337). Although the effect of *Taq* on the results must be considered, there was evidence that it did not have a significant effect. For example, GS7 and GS8 had been isolated on the same day from the same sample site and therefore it was likely that they would be very similar in sequence. In fact, the sequencing of GS7 and GS8 showed that there was complete sequence homology between these two viruses in the HA1 region and the M2 transmembrane domain (Figure 6.4 and Table 6.3). Therefore, this demonstrated that the effects of *Taq* had not cause the appearance of mutations in viruses that were expected to be very similar in sequence. Additionally, the cloning of GS1 and GS10 had shown that the GS10 clones had little variation within the HA1 region (Appendix 25 and 26). This provided further

evidence that the effects of *Taq* within this study had not been detrimental to the results.

6.5.5 General Conclusions

In summary, the rapid development of drug-resistant following a short course of amantadine treatment illustrated the limitations of amantadine, especially in the immunocompromised host. It was interesting to note the apparent selective advantage that the resistant population maintained over the sensitive for a long period of time in the absence of drug selective pressure. The study revealed the presence of complex viral populations undergoing constant fluctuations, possibly as a direct result of selective pressures of "intra-population" competition. This was illustrated by the changing drug genotype found throughout the study period and the presence of mixed variants within the virus population. It was hypothesised that over the study period, the relative fitness of the viral population, in respect to adaptation to the host, had increased as indicated by evidence from inoculation of the first and last viruses into ferrets. This was also supported by antigenic profiles of the viruses that illustrated the gradual changes and accumulation of mutations over the study period, antigenic drift.

This work illustrated the importance of studying virus population dynamics in individuals, and how the immunocompromised host represents an invaluable source of data concerning the progression of persistent influenza infections.

Chapter 7

General Conclusions

7.1 Amantadine-Resistance in Natural Isolates and an Immunocompromised Host

This project investigated the emergence and evolution of amantadine-resistant viruses in the UK population and an individual immunocompromised host. The characterisation of each set of resistant viruses revealed some interesting features. Resistance within the population appeared to occur sporadically and often in clusters. There was evidence from phenotypic, genotypic and phylogenetic analyses to suggest that some of these clusters were actually small outbreaks that had occurred as a direct consequence of amantadine use. The available data suggested that there was no persistence of resistant strains in the population. Seasons containing resistant viruses were often foilowed by a season(s) where no resistance could be detected. This might have been due to the lack of drug selective pressure acting on the resistant viruses, not enabling them to compete with wild-type sensitive strains. However, in the immunocompromised host (GS), resistant viruses were maintained in a relatively long persistent infection in the absence of any selective drug pressure.

When comparing the evolution of influenza through changes occurring in antigenic regions in the normal and immunocompromised host, it is important to remember the selective pressures targeting the viral surface antigens involved in each system. In the immunocompetent host, the immune system, consisting of both humorai and cell-mediated responses, manages to clear infectious virus from the host within 7-10 days after initial infection. Therefore, the time that the virus has in the immunocompetent host is relatively limited. It is possible that the antigenic changes required to evade the immune system of susceptible hosts are acquired over a number of infectious episodes i.e. mutations are accumulated over time. Therefore, it would appear that the evolution within susceptible groups does not occur exclusively through individual hosts. Instead, a virus infecting a series of susceptible hosts is exposed to a certain level of immune pressure during each infection. This continual pressure selects variants with changes in antigenic regions that gradually alters the overall antigenicity of the virus. This process enables the virus to eventually evade immune detection by changing the profile of its' antigenic "cloak".

In contrast, the immune system present in GS was severely compromised and therefore there was no significant clearance of virus from the host, which resulted in the persistent infection. Evidence has been gathered to suggest that the invading virus was not under any detectable selective pressure, yet evidence for apparent antigenic drift within this set of viruses was observed. Unlike the viruses circulating the general population where immune pressure was driving change, in GS it was possible that other forces were causing the observed changes. One possible reason for this was the selection of variants that displayed more adaptation to the host. This could have selected for variants containing changes in receptor-binding sites, altering the affinity for different host-cell receptors, giving those variants a selective advantage. This gradual selection of variants could have been responsible for the overall changes observed in antigenicity. Therefore, it was possible that the hypothesised drive to adapt to the host induced the selection of variants that had antigenic changes and gave the appearance of drift. In the absence of immune pressure it was thought that the virus would have been able to replicate unhindered, as suggested by the rate of evolution determined in the HA1 region of the viruses. It was thought that this coupled with a continual drive to select variants most adapted to the host, probably resulted in the observed results. It was possible that the overall consequence of this selection procedure would have been that the final virus (GS10) would have been highly adapted to the host when compared to the first isolate (GS1) that initiated the infection. Evidence to support this was seen when the above viruses were inoculated into ferrets; the first virus replicated extremely efficiently but the last virus appeared to struggle, i.e. it was highly adapted to GS, but when introduced into a different environment it lost the relative fitness gains accumulated over the persistent infection. Therefore, a comparison of the two sets of data demonstrated continual and combined host-driven immune pressure in the normal population, and interspecies-driven competition to adapt to the conditions in the immunocompromised host.

In relation to amantadine-resistance, similar comparisons between the population and individual host could be made. In immunocompetent hosts there was little evidence for transmission, and thus persistence of amantadine-resistance suggesting that resistant variants were at a selective disadvantage. In GS, the continued selection of resistance for a long period of time in the absence of drug selective pressure suggested that in this situation the resistant variants were not at a selective disadvantage. It was possible that in GS, the proposed higher

replication rate of the viruses might have enabled the resistant population to maintain higher numbers and therefore remain dominant over time. In a host where an active immune system suppressed replication, it was possible that this selective pressure might have favoured the wild-type sensitive variants resulting in a swing back to a sensitive phenotype. In this scenario, it would be interesting to perform an experiment to simulate the conditions observed in this study. An immunocompetent and immunosuppressed animal model could be infected with an amantadine-sensitive influenza virus. A short course of amantadine treatment would possibly select a resistant population that could be detected by sampling and determining the drug susceptibility of the viruses. The amantadine treatment could then be stopped and the progress of infection monitored to see if the resistant population reverted back to the sensitive wild-type in either set of animals. This type of experiment could also be repeated using two groups of susceptible animals, again competent and suppressed to see if transmission of resistant strains in the absence of drug results in the virus reverting back to the sensitive wild-type. The data that this proposed experiment would produce could increase the understanding of how immune pressure might affect the selection of amantadine-resistant and -sensitive variants.

7.2 Influenza and HIV Antiviral Drug-Resistance

The development of antiviral drugs has revolutionised patient management and the treatment of viral diseases including those caused by influenza, HIV, herpes and hepatitis viruses. In contrast to the development of a relatively small number of influenza antiviral compounds over the past thirty years, there is a broad range of drugs availabie for targeting various stages of the HIV replication cycle. However, resistance to anti-retroviral drugs of all classes has been documented, especially with the nucleoside analogue reverse transcriptase inhibitors and protease inhibitors (155). The frequency of amantadine-resistance has been estimated to be approximately 1-2% in natural influenza A isolates (85, 417), and 30-60% in patients undergoing amantadine treatment (147, 238). Although the frequency of HIV infection varies considerably world wide, in Europe and the US the frequency of HIV drug-resistance it is thought to be in the range of 10-25% (32, 221, 405). This figure is more comparable with influenza A resistance in immunocompromised patients. A common factor in these relatively high figures

might be that patients associated with these levels of resistance generaily have depleted immune systems.

There are several similarities between the emergence of drug-resistance in influenza and HIV. Both are RNA viruses, undergo rapid genetic change and exist as heterogeneous populations (quasispecies). Therefore, both viruses have the potentiai to rapidly select drug-resistant mutants and establish resistant populations within the host. Studies have provided evidence for the transmission of amantadine-resistant influenza variants between hosts (22, 147). Comparative studies of amantadine-sensitive and -resistant influenza A viruses have shown that in certain models, resistant variants potentially have equal levels of virulence as the sensitive strains (350). The documented transmission of drug-resistant HIV-1 viruses also suggests that they may also possess equal levels of virulence when compared to sensitive populations (155, 172, 370, 405). Multi-drug resistant HiV-1 strains have also been shown to transmit between hosts (155). It would appear however that certain HIV-1-resistant mutations might reduce the replication rate and fitness of the virus (17). Drug-resistant viruses carrying mutations within the reverse transcriptase (RT) gene were shown to have reduced RT enzyme activity that resulted in the virus having a lower rate of replication. It would appear that this form of mutation might reduce the efficiency of transmission and decrease the potential for this resistant mutant to spread to other susceptible hosts (17). Therefore, transmission of resistant viruses, both influenza and HIV probably involves resistant mutants with the highest levels of fitness in respect to replicative potential.

The overall implication of transmissible drug-resistant mutants is extremeiy worrying from a pubiic health perspective. This could potentially result in treatment-naïve patients acquiring an infection for which there is no effective drug therapy avaiiable. Presently, for influenza this only has serious consequences for at-risk groups where influenza related mortality is highest. However, if an epidemic or pandemic strain possessing the same virulence as seen in the 1918 pandemic was to emerge, drug prophylaxis or therapy would probably represent the only method to combat the spread of disease. To imagine that widespread drug treatment could result in the appearance of resistant, transmissible strains, rendering uninfected hosts susceptible and untreatable, illustrates the seriousness of this situation.

7.3 Virus Ion Channels

The influenza A M2 ion channel represents the most intensively studied of all viral ion channels. From previous work, there is now a good understanding of the processes involved in the antiviral activities of amantadine and rimantadine and the overali importance of the ion channel in the replication cycle of the virus (142). Other viral ion channels studied include the influenza B and C channels, NB and CM2 respectively (205, 276). Also, two HIV-1 proteins have been implicated in having channel activity, Vpu and Vpr (94, 205, 280).

The presence of an ion channel may highlight the presence of a possible target for antiviral compounds, as shown with influenza A and amantadine. However, amantadine can only inhibit influenza B at millimolar concentrations when the drug actually acts as a lysosomaltrophic agent, raising the lumenal pH of intracellular compartments. Electrophysiological studies of Vpu expressed in different cell systems have shown that after applying Vpu to bilayers, permeability to selected cations was recorded (205). This represents good evidence for the presence of ion channel activity associated with the Vpu protein. The function of this protein is to regulate the release of virus particles from HIV-1 infected cells (357). Therefore, this protein would seem to represent a good target to formulate a compound that would inhibit channel activity and therefore limit the release of progeny virus particles.

7.4 Future Work

Although proposed future work has been outlined in each respective chapter, there are some important areas that required completion to finalise parts of the project. The finding that some phenotypically resistant viruses lacked M2 transmembrane mutations requires further analysis. It was proposed that the observed results might have been due to the presence of quasispecies within the virus isolates. This might have resulted in the RT-PCR amplification of sensitive amplicons instead of resistant. However, it has been previously shown that resistant viruses can have a wild-type sensitive M2 protein, compensated by mutations within the HA2 molecule (340). Therefore, it would be interesting to sequence the HA2 region of this set of resistant viruses to see if they possessed this mutation. If it

were found that some of the resistant viruses did have HA2 mutations, this would have important implications. The previous studies were performed using avian viruses, where the HA and M2 proteins have a close relationship in the replication cycle (346). The viruses sequenced in this study were exclusively human in origin, therefore if mutations in the HA2 molecule were conferring amantadine-resistance, this might imply that the HA within these viruses had been altered its overall structure or function to behave more avian-like.

It has been previously suggested that the growth potential and virulence of amantadine-resistant strains can be dependent on the M2 transmembrane mutation conferring the resistance (118, 350). It was proposed that a simple reverse genetics system be designed and implemented to clone gene segment seven of a resistant virus into a sensitive wild-type virus. The resulting virus could be rescued and analysed by growth curve assays *in vitro* to assess the importance of M1 and M2 from resistant viruses in controlling growth. The results from these experiments could provide further data to possibly explain why certain M2 mutations occur commonly in human resistant strains e.g. Ser31Asn, while some are extremely rare or cannot be detected e.g. Gly34Glu. This technique could also be used to assess the effects of novel mutations found in the present study. These mutations have not previously been reported in human influenza A viruses therefore data regarding their effect on viral replication and function of gene segment would be vital.

Previous studies have demonstrated that different shapes of the influenza virion i.e. spherical or filamentous can influence the viral yields and plaque sizes of the viruses in question (302). In this present study, it was hypothesised that the GS viruses isolated from an immunosuppressed child displayed continual adaptation to the host resulting in the final virus isolate (GS10) to be the most highly adapted isolate. Therefore, it was proposed that several GS viruses could be investigated for particle shape. This would reveal any correlation between particle shape and the relative level of fitness that viruses had acquired to GS. In addition, it is proposed that a selection of amantadine-resistant viruses with different drug genotypes are analysed for particle shape. The M1 and M2 proteins have been implicated in controlling particle shape, therefore it would be interesting to investigate whether amantadine-resistant mutations within M2 alter the morphology of the virion. Resuits from this work might possibly provide a further

explanation for the apparent dominance of certain human amantadine-resistance mutations, i.e. if resistance confers particle shape that increases virus yield, this might increase the fitness of the resistant genotype and therefore its' prevalence.

Although the majority of work in this study has concentrated on the functional properties of M2, the data accumulated regarding M1 also has great importance for future work. M1 has previously been implicated in controlling rapid virus growth in certain influenza strains (402, 404). Results from Chapter 3 demonstrated the variation in infectivity titres (PFU/ml) of viruses tested. The sequencing of the M1 gene from a large number influenza A viruses in this study provides an ideal opportunity to relate observed molecular differences with the relative growth rates of the viruses. This type of data might reveal M1 mutations that are responsible for altering the growth properties of viruses. The relevance of this proposed work is associated with developing annual human influenza vaccines. Current research is developing methods to change the production of influenza vaccines from eggs to tissue culture (270, 373). Growing vaccine strains in eggs has many problems and also prevents individuals with specific albumen allergies from receiving the vaccine (248). Growth of vaccine strains in tissue culture also presents problems, generally viruses grow at much iower titres in tissue culture than eggs. Therefore, investigating the potential influence of M1 on viral growth has important implications for future vaccine work. If M1 or M2 containing the relevant motifs required for high growth could be packaged into a parental virus backbone presenting the required surface glycoproteins, then the recombinant virus could be grown to high titres in tissue culture and used to create vaccine preparations.

The current work has demonstrated the methods that are used to determine the susceptibility of influenza A viruses to amantadine. Viruses were tested by phenotypic methods and those viruses that were determined resistant were then characterised genetically to identify resistance markers. With the predicted future increased use of NI drugs, it is important to set up similar protocols for the testing of virus susceptibility to these new drugs. Although similar methodologies can be used, there are several difficulties when testing the susceptibility of influenza viruses to NI drugs. In previous studies using traditional *in vitro* susceptibility assays, there has been considerable variation observed in the susceptibility of viruses in the plaque reduction assay (PRA) (20, 126). Additionally, viruses that appeared to have reduced sensitivity to NI drugs *in vitro* (PRA) were highly

sensitive to drug in the ferret model (20). Therefore, it appears that susceptibility testing for NI drugs requires a modified approach. The widely accepted method for predicting the sensitivity of viruses to NI drugs *in vivo* is the NA assay. This measures enzymatic activity of the molecule and is usually confirmed with HA sequencing (43, 126).

Therefore, future work should involve the implementation of protocols and screening for both amantadine and NI drug susceptibility in the routine laboratory setting. It will be interesting to observe the frequency of resistance to NI drugs occurring in the general population especially during an influenza season within high levels of activity. The development of resistance has not been predicted, however the genetic variability of the influenza virus almost makes it a forgone conclusion that it will occur. If both drugs were used in combined therapy as previously described (211), it would be interesting to screen viruses for dual resistance markers. Influenza strains resistant to both ciasses of drug would represent a serious problem as has been observed with other multi-drug resistant organisms e.g. *Staphylococcus aureus* and HIV (117, 155, 356). Therefore, work should be performed to assess the predicted virulence, transmissibility, fitness etc. of these theoretical strains to ensure that maximal protection can be afforded to patients at risk from infection.

7.5 Concluding Remarks

The surveillance of antiviral susceptibility is a fundamental requirement for understanding the mechanisms responsible for emerging resistant virus strains. The genetic makeup of influenza A viruses is continually changing, a feature that enabies the virus to generate resistant mutants with relative ease. This study was designed to assess the impact of antiviral drug use in the UK by testing the susceptibility of influenza A virus strains to amantadine. This demonstrated low levels of resistance to amantadine and provided evidence to suggest that these viruses were not readily transmitted within the population. Sporadic clusters of resistant viruses within certain influenza seasons could be linked with evidence suggesting that they had originated in the institutional setting, e.g. boarding schools.

With the recent advent of novel NI drugs, the future clinical usefulness of amantadine and rimantadine in the treatment of influenza has been put in doubt. However, at present the relative number of ciinical studies demonstrating the use of these NI inhibitors is iimited and the clinical effectiveness and safety has not been substantiated in some at-risk groups. There have been numerous controlled studies using amantadine and rimantadine on all patient groups demonstrating their ability to prevent and treat iliness (37, 74, 149, 165), and therefore these drugs still appear to be preferential when treating certain patients. Cost is aiso a major consideration when prescribing influenza inhibitors. A course of amantadine treatment is approximately one fifth the cost of an equivalent course of zanamivir, making the former drug much more economicaliy viable. Another consideration is the relative shelf life of each inhibitor compound. Amantadine and rimantadine have been shown to retain their antiviral potency for up to thirty years (319). Similar data for NI drugs has not been possible due to their relative novelty. The relevance of this point is illustrated when planning for future pandemic situations. A stable drug with a long shelf life can easily be stockpiled for future use in combating wide scale disease.

The above discussion demonstrates some of the advantages that amantadine and rimantadine have over NI inhibitors. There are of course many disadvantages as previously mentioned in this thesis with anti-M2 protein drugs, including the generation of resistant mutants. However, it might be suggested that combined treatment and prophylaxis using both classes of drug under certain situations might actually represent the most efficient method of controlling the spread of influenza where vaccination has not been implemented or has failed (212).

Although the advent of NI drugs has provided a new approach to the provision of protection against influenza infections, the nature of the influenza virus requires that continuing research into future drugs should continue. Presently, new NI drugs continue to be developed, each containing novel structural alterations improving their antiviral potency e.g. RWJ-270201 and ABT-675 (178, 407). However, focusing research on this single protein might increase the selective pressure exerted and thus increase the probability of resistant mutants emerging through drug prophylaxis and treatment. Mutations within the NA molecule may indeed eventually provide complete cross-resistance to ali NI drugs, mutations have aiready been identified *in vitro* that reduce sensitivity of influenza to two NI

inhibitors (352). Therefore, it is vital to maintain continued surveillance of drug susceptibility on a national and global scale and within certain at-risk groups, e.g. the immunocompromised, where the emergence of unique viral infections can be studied.

References

1. **Ait-Khaled, M., J. E. McLaughlin, M. A. Johnson, and V. C. Emery.** 1995. Distinct HIV-1 long terminal repeat quasispecies present in nervous tissues compared to that in lung, blood and lymphoid tissues of an AIDS patient. AIDS. **9:**675-683.
2. **Aldrich, P. E., E. C. Hermann, W. E. Meier, M. Paulshock, W. W. Prichard, J. A. Snyder, and J. C. Watts.** 1971. Antiviral agents 2. Structure-activity relationships of compounds related to 1-adamantanamine. Journal of Medicinal Chemistry. **14:**535-543.
3. **Aiford, R. H., J. A. Kasei, P. J. Gerone, and V. Knight.** 1966. Human influenza resulting from aerosol inhalation. Proceedings of the Society of Experimental Biology and Medicine. **122:**800-804.
4. **Alien, H., J. McCauley, M. Waterfield, and M. J. Gething.** 1980. Influenza virus RNA segment 7 has the coding capacity for two polypeptides. Virology. **107:**548-551.
5. **Altmuller, A., W. M. Fitch, and C. Schoitissek.** 1989. Biological and genetic evolution of the nucleoprotein gene of human influenza A viruses. Journal of General Virology. **70:**2111-2119.
6. **Anon.** 1977. Influenza: The Birmingham Research Unit of the Royal College of General Practitioners. Journal of the Royal College of General Practitioners. **27:**544-551.
7. **Anon.** 2000. Prevention and control of influenza: recommendations of the Advisory Committee on Immunisation Practices (ACIP). Morbidity and Mortality Weekly Report. **49:**6-7.
8. **Aoki, F. Y.** 1998. Amantadine and rimantadine, p. 457-476. *In* K. G. Nicholson and R. G. Webster and A. J. Hay (ed.), Textbook of Influenza. Blackwell Sciences, Oxford.
9. **Apalsch, A. M., M. Green, J. Ledesma-Medina, B. Nour, and E. R. Wald.** 1995. Parainfluenza and influenza virus infections in paediatric organ transplant recipients. Clinical Infectious Dlseases. **20:**394-399.
10. **Appleyard, G.** 1977. Amantadine-resistance as a genetic marker for influenza viruses. Journal of General Virology. **36:**249-255.
11. **Appleyard, G., and H. B. Maber.** 1975. A plaque assay for the study of influenza virus inhibitors. Journal of Antimicrobial Chemotherapy. **1:**49-53.

12. **Arbour, N., G. Cote, C. Lachance, M. Tardieu, N. R. Cashman, and P. J. Talbot.** 1999. Acute and persistent infection of human neural cell lines by human coronavirus OC43. Journal of Virology. **73:**3338-3350.

13. **Arbour, N., S. Ekande, G. Cote, C. Lachance, F. Chagnon, M. Tardieu, N. R. Cashman, and P. J. Talbot.** 1999. Persistent infection of human oligodendrocytic and neuroglial cell lines by human coronavirus 229E. Journal of Virology. **73:**3326-3337.

14. **Arden, N. H., P. A. Patiarea, M. B. Fasano, K. J. Lui, M. W. Harmon, A. P. Kendal, and D. Rimland.** 1988. The roles of vaccination and amantadine prophylaxis in controlling an outbreak of influenza A (H3N2) in a nursing home. Archives of Internal Medicine. **148:**865-868.

15. **Atkins, M., P. Strappe, S. Kaye, C. Loveday, J. E. McLaughlin, M. A. Johnson, R. S. Tedder, P. D. Griffiths, and V. C. Emery.** 1998. Quantitative differences in the distribution of zidovudine resistance mutations in multiple post-mortem tissues from AIDS patients. Journal of Medical Virology. **55:**138-146.

16. **Babu, Y. S., P. Chand, S. Bantia, P. Kotian, A. Dehghani, Y. El-Kattan, T. H. Lin, T. L. Hutchison, A. J. Elliott, C. D. Parker, S. L. Ananth, L. L. Horn, G. W. Laver, and J. A. Montgomery.** 2000. BCX-1812 (RWJ-270201): discovery of a novel, highly potent, orally active, and selective influenza neuraminidase inhibitor through structure-based drug design. Journal of Medicinal Chemistry. **43:**3482-3486.

17. **Back, N. K., M. Nijhuis, W. Keulen, C. A. Boucher, B. O. Oude Essink, A. B. van Kuilenburg, A. H. van Gennip, and B. Berkhout.** 1996. Reduced replication of 3TC-resistant HIV-1 variants in primary cells due to a processivity defect of the reverse transcriptase enzyme. EMBO Journal. **15:**4040-4049.

18. **Barnett, B. C., D. S. Burt, C. M. Graham, A. P. Warren, J. J. Skehel, and D. B. Thomas.** 1989. I-Ad restricted T celi recognition of influenza hemagglutinin. Synthetic peptides identify multiple epitopes corresponding to antibody-binding regions of the HA1 subunit. Journal of Immunology. **143:**2663-2669.

19. **Barnett, B. C., I. Hartlmayr, C. M. Graham, and D. B. Thomas.** 1990. Single amino acid residues in a synthetic peptide of influenza haemagglutinin, HA 1 177-199, distinguish I-Ad- and I-Ed-restricted T-celi epitopes. Immunology. **70:**48-54.

20. **Barnett, J. M., A. Cadman, D. Gor, M. Dempsey, M. Walters, A. Candlin, M. Tisdale, P. J. Morley, I. J. Owens, R. J. Fenton, A. P. Lewis, E. C. Claas, G. F. Rimmelzwaan, R. De Groot, and A. D. Osterhaus.** 2000. Zanamivir susceptibility monitoring and characterization of influenza virus clinical Isolates obtained during phase II clinical efficacy studies. Antimicrobial Agents and Chemotherapy. **44:**78-87.

21. **Basler, C. F., A. H. Reid, J. K. Dybing, T. A. Janczewski, T. G. Fanning, H. Zheng, M. Salvatore, M. L. Perdue, D. E. Swayne, A. Garcia-Sastre, P. Palese, and J. K. Taubenberger.** 2001. Sequence of the 1918 pandemic influenza virus nonstructural gene (NS) segment and characterisation of recombinant viruses bearing the 1918 NS genes. Proceedings of the National Academy of Sciences USA. **98:**2746-2751.

22. **Bean, W. J., S. C. Threlkeld, and R. G. Webster.** 1989. Biological potential of amantadine-resistant influenza A virus in an avian model. Journal of Infectious Diseases. **159:**1050-1056.

23. **Belshe, R. B., B. Burk, F. Newman, R. L. Cerruti, and I. S. Sim.** 1989. Resistance of influenza A virus to amantadine and rimantadine: resuits of one decade of surveillance. Journal of Infectious Diseases. **159:**430-5.

24. **Belshe, R. B., P. M. Mendelman, J. Treaner, J. King, W. C. Gruber, P. Piedra, D. I. Bernstein, F. G. Hayden, K. Kotloff, K. Zangwill, D. Iacuzio, and M. Wolff.** 1998. The efficacy of live attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine in children. New England Journal of Medicine. **338:**1405-1412.

25. **Belshe, R. B., M. H. Smith, C. B. Hail, R. Betts, and A. J. Hay.** 1988. Genetic basis of resistance to rimantadine emerging during treatment of influenza virus infection. Journal of Virology. **62:**1508-1512.

26. **Betts, R. F., J. J. Treanor, P. S. Graman, D. W. Bently, and R. Dolin.** 1987. Antiviral agents to prevent or treat influenza in the elderly. Journal of Respiratory Disease. **8:**S56-59.

27. **Biswas, S. K., P. L. Boutz, and D. P. Nayak.** 1998. Influenza virus nucleoprotein interacts with influenza virus polymerase proteins. Journal of Virology. **72:**5493-5501.

28. **Blaas, D., E. Patzelt, and E. Kuechler.** 1982. Cap-recognising protein of influenza virus. Virology. **116:**339-348.

29. **Black, R. A., P. A. Rota, N. Gorodkova, H. D. Klenk, and A. P. Kendal.** 1993. Antibody response to the M2 protein of influenza A virus expressed in insect cells. Journal of General Virology. **74:**143-146.

30. **Blick, T. J., A. Sahasrabudhe, M. McDonald, I. J. Owens, P. J. Morley, R. J. Fenton, and J. L. McKimm-Breschkin.** 1998. The interaction of neuraminidase and haemagglutinin mutations in influenza virus in resistance to 4-Guanidino-Neu5Ac2en. Virology. **246:**95-103.

31. **Blick, T. J., T. Tiong, A. Sahasrabudhe, J. N. Varghese, P. M. Colman, G. J. Hart, R. C. Bethell, and J. L. McKimm-Breschkin.** 1995. Generation and characterisation of an infiuenza virus neuraminidase variant with decreased sensitivity to the neuraminidase-specific inhibitor 4-guanidino-Neu5Ac2en. Virology. **214:**475-484.

32. **Boden, D., A. Hurley, L. Zhang, Y. Cao, Y. Guo, E. Jones, J. Tsay, J. Ip, C. Farthing, K. Limoli, N. Parkin, and M. Markowitz.** 1999. HIV-1 drug-resistance in newly infected individuals. Journal of the American Medical Association. **282:**1135-1141.

33. **Boom, R., C. J. Soi, M. M. Saiimans, C. L. Jansen, P. M. Wertheim-van Dillen, and J. van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. Journai of Clinical Microbiology. **28:**495-503.

34. **Both, G. W., M. J. Sleigh, N. J. Cox, and A. P. Kendal.** 1983. Antigenic drift in influenza virus H3 haemagglutinin from 1968 to 1980: multiple evolutionary pathways and sequential amino acid changes at key antigenic sites. Journal of Virology. **48:**52-60.

35. **Braam, J., I. Ulmanen, and R. M. Krug.** 1983. Molecular model of a eucaryotic transcription complex: functions and movements of influenza P proteins during capped RNA-primed transcription. Cell. **34:**609-618.

36. **Bracho, M. A., A. Moya, and E. Barrio.** 1998. Contribution of *Taq* polymerase-induced errors to the estimation of RNA virus diversity. Journal of General Virology. **79:**2921-2928.

37. **Brady, M. T., S. D. Sears, D. L. Pacini, R. Samorodin, J. DePamphilis, M. Oakes, W. Soo, and M. L. Clements.** 1990. Safety and prophylactic efficacy of low-dose rimantadine in adults during an influenza A epidemic. Antimicrobial Agents and Chemotherapy. **34:**1633-1636.

38. **Brassard, D. L., G. P. Leser, and R. A. Lamb.** 1996. Influenza B virus NB glycoprotein is a component of the virion. Virology. **220:**350-360.

39. **Bukrinskaya, A. G., N. K. Vorkunova, G. V. Kornilayeva, R. A. Narmanbetova, and G. K. Vorkunova.** 1982. Influenza virus uncoating in infected cells and effect of rimantadine. Journal of General Virology. **60.**

40. **Bukrinskaya, A. G., N. K. Vorkunova, and N. L. Pushkarskaya.** 1982. Uncoating of a rimantadine-resistant variant of infiuenza virus in the presence of amantadine. Journal of General Virology. **60:**61-66.

41. **Buonagurio, D. A., S. Nakada, J. D. Parvin, M. Krystal, P. Palese, and W. M. Fitch.** 1986. Evolution of human influenza A viruses over 50 years: rapid, uniform rate of change in NS gene. Science. **232:**980-982.

42. **Burt, D. S., K. H. Mills, J. J. Skehel, and D. B. Thomas.** 1989. Diversity of the class II (I-Ak/I-Ek)-restricted T cell repertoire for influenza hemagglutinin and antigenic drift. Six nonoverlapping epitopes on the HA1 subunit are defined by synthetic peptides. Journal of Experimental Medicine. **170:**383-397.

43. **Buxton, R. C., B. Edwards, R. R. Juo, J. C. Voyta, M. Tisdale, and R. C. Bethell.** 2000. Development of a sensitive chemiluminescent neuraminidase assay for the determination of influenza virus susceptibility to zanamivir. Analytical Biochemistry. **280:**291-300.

44. **Carr, C. M., C. Chaudhry, and P. S. Kim.** 1997. Influenza haemagglutinin is spring-loaded by a metastable native conformation. Proceedings of the National Academy of Sciences USA. **94:**14306-14313.

45. **Castrucci, M. R., and Y. Kawaoka.** 1995. Reverse genetics system for generation of an influenza A virus mutant containing a deletion of the carboxyl-terminal residue of M2 protein. Journal of Virology. **69:**2725-2728.

46. **Chizhmakov, I. V., F. M. Geraghty, D. C. Ogden, A. Hayhurst, M. Antoniou, and A. J. Hay.** 1996. Selective proton permeability and pH regulation of the influenza virus M2 channel expressed in mouse erythroleukaemia cells. Journal of Physiology. **494:**329-336.

47. **Choppin, P. W., and R. W. Compans.** 1975. The structure of influenza virus, p. 15-47. *In* E. D. Kilbourne (ed.), The Influenza Viruses and Influenza. Academic Press, New York.

48. **Claas, E. C., A. D. Osterhaus, R. van Beek, J. C. De Jong, G. F. Rimmelzwaan, D. A. Senne, S. Krauss, K. F. Shortridge, and R. G. Webster.** 1998. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet. **351:**472-477.

49. **Clavo, A. C., H. F. Maassab, and M. W. Shaw.** 1993. A persistent infection in MDCK cells by an influenza type B virus. Virus Research. **29:**21-31.

50. **Cline, J., J. C. Braman, and H. H. Hogrefe.** 1996. PCR fidelity of *Pfu* DNA polymerase and other thermostable DNA polymerases. Nucleic Acids Research. **24:**3546-3551.

51. **Cochran, K. W., H. F. Maassab, A. Tsunoda, and B. S. Berlin.** 1965. Studies on the antiviral activity of amantadine hydrochloride. Annals of the New York Academy of Sciences. **130:**432-439.

52. **Cockburn, W. C., P. J. Delon, and W. Ferreira.** 1969. Origin and progress of the 1968-69 Hong Kong influenza epidemic. Bulletin of the World Health Organisation. **41:**345-348.

53. **Colman, P. M.** 1989. Influenza virus neuraminidase: enzyme and antigen, p. 175-218. *In* R. M. Krug (ed.), The Influenza Viruses. Plenum Press, New York.

54. **Coiman, P. M., J. N. Varghese, and W. G. Laver.** 1983. Structure of the catalytic and antigenic sites in influenza virus neuraminidase. Nature. **303:**41-44.

55. **Conne, P., L. Gauthey, P. Vernet, B. Althaus, J. U. Que, B. Finkel, R. Giück, and S. J. Cryz.** 1997. Immunogenicity of trivalent subunit versus virosome-formulated influenza vaccines in geriatric patients. Vaccine. **15:**1675-1679.

56. **Connolly, A. M., R. L. Salmon, B. Lervey, and D. H. Williams.** 1993. What are the complications of influenza and can they be prevented? Experience from the 1989 epidemic of H3N2 influenza A in general practice. British Medical Journal. **306:**1452-1454.

57. **Connor, R. J., Y. Kawaoka, R. G. Webster, and J. C. Paulson.** 1994. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. Virology. **205:**17-23.

58. **Corey, L., R. J. Rubin, M. A. W. Hattwick, G. R. Nobel, and E. Cassidy.** 1976. A nationwide outbreak of Reye's syndrome: its epidemiologic relationship to infiuenza B. American Journal of Medicine. **61:**615-625.

59. **Cox, N. J., and C. A. Bender.** 1995. The moiecular epidemiology of influenza viruses. Seminars in Virology. **6:**359-370.

60. **Cox, N. J., and K. Subbarao.** 1999. Influenza. Lancet. **354:**1277-1282.

61. **Crawford, S. A., R. D. Clover, T. D. Abell, C. N. Ramsey, P. Glezen, and R. B. Couch.** 1988. Rimantadine prophylaxis in children: a follow-up study. Pediatric Infectious Disease Journal. **7:**379-383.

62. **Crosby, A.** 1989. America's Forgotten Pandemic. Cambridge University Press, Cambridge.

63. **Daniels, R. S., J. J. Skehel, and D. C. Wiley.** 1985. Amino acid sequences of haemagglutinins of influenza viruses of the H3 subtype isolated from horses. Journal of General Virology. **66:**457-464.

64. **Davies, J. R., E. A. Grilli, A. J. Smith, and T. W. Hoskins.** 1988. Prophylactic use of amantadine in a boarding school outbreak of influenza A. Journal of the Royal College of General Practitioners. **38:**346-348.

65. **Davies, W. L., R. R. Grunert, R. F. Haff, J. W. McGahne, E. M. Neumayer, M. Paulshock, J. C. Watts, E. C. Hermann, and C. E. Hoffman.** 1964. Antiviral activity of 1-adamantanamine (amantadine). Science. **144:**862-863.

66. **Dedman, D., C. A. Joseph, P. Chakraverty, and J. M. Watson.** 1993. Influenza surveillance, England and Wales: October 1992 - June1993. Communicable Disease Report Review. **3:**R184-186.

67. **Dedman, D. J., C. A. Joseph, P. Chakraverty, D. M. Fleming, and J. M. Watson.** 1994. Influenza surveillance, England and Wales: October 1993 - June1994. Communicable Disease Report Review. **4:**R164-168.

68. **Dedman, D. J., C. A. Joseph, M. Zambon, D. M. Fleming, and J. M. Watson.** 1997. Influenza surveillance in England and Wales: October 1996 - June 1997. Communicable Disease Report Review. **7:**R212-219.

69. **Dedman, D. J., and J. M. Watson.** 1997. The use of thresholds to describe leveis of influenza activity. PHLS Microbiology Digest. **14:**206-208.

70. **Dedman, D. J., M. Zambon, P. Van Buynder, D. M. Fleming, J. M. Watson, and C. A. Joseph.** 1998. Influenza surveillance in England and Wales: October 1997 - June 1998. Communicable Disease and Public Health. **1:**244-251.

71. **Degelau, J., S. K. Somani, S. L. Cooper, D. R. P. Guay, and K. B. Crossley.** 1992. Amantadine-resistant influenza A in a nursing home. Archives of Internal Medicine. **152:**390-392.

72. **Demicheli, V., T. Jefferson, D. Rivetti, and J. Deeks.** 2000. Prevention and treatment of influenza in healthy adults. Vaccine. **18:**957-1030.

73. **Digard, P., V. C. Blok, and S. C. Inglis.** 1989. Complex formation between influenza virus polymerase proteins expressed in *Xenopus* oocytes. Virology. **171:**162-169.

74. **Dolin, R., R. C. Reichman, H. P. Madore, R. Maynard, P. N. Linton, and J. Webber-Jones.** 1982. A controlled trial of amantadine and rimantadine in the prophylaxis of influenza A infection. New England Journal of Medicine. **307:**580-584.

75. **Domingo, E.** 1996. Biological significance of viral quasispecies. Viral Hepatitis. **2:**247-261.

76. **Domingo, E., J. Díez, M. A. Martínez, J. Hernández, A. Holguín, B. Borrego, and M. G. Mateu.** 1993. New observations on antigenic diversification of RNA viruses: antigenic variation is not dependent on immune selection. Journal of General Virology. **74:**2039-2045.

77. **Domingo, E., and J. J. Holland.** 1994. Mutation rates and rapid evolution of RNA viruses, p. 161-184. *In* S. S. Morse (ed.), The Evolutionary Biology of Viruses. Raven Press Ltd, New York.

78. **Domingo, E., L. Menvéndez-Arias, and J. J. Holland.** 1997. RNA virus fitness. Reviews in Medical Virology. **7:**87-96.

79. **Duff, K. C., and R. H. Ashley.** 1992. The transmembrane domain of influenza A M2 protein forms amantadine-sensitive proton channels in planar lipid bilayers. Virology. **190:**485-489.

80. **Duncan, G., and F. Colbere-Garapin.** 1999. Two determinants in the capsid of a persistent type 3 poliovirus exert different effects on mutant virus uncoating. Journal of General Virology. **80:**2601-2605.

81. **Dunn, F. L.** 1958. Pandemic of influenza in 1957. Journal of the American Medical Association. **166:**1140-1148.

82. **Edmond, J. D., R. G. Johnston, D. Kidd, H. J. Rylance, and R. G. Sommerville.** 1966. The inhibition of neuraminidase and antiviral action. British Journal of Pharmacological Chemotherapy. **27:**415-426.

83. **Eigen, M., W. Gardiner, P. Schuster, and R. Winkler-Oswatitsch.** 1981. The origin of genetic information. Scientific American. **244:**78-94.

84. **Eisen, M. B., S. Sabesan, J. J. Skehel, and D. C. Wiley.** 1997. Binding of the influenza A virus to cell-surface receptors: structures of five hemagglutinin-sialyloiigosaccharide complexes determined by X-ray crystallography. Virology. **232:**19-31.

85. **Elliot, A. J., and M. C. Zambon.** 2001. Amantadine susceptibility screening of influenza A isolates in the UK (1958-1999). Antimicrobial Agents and Chemotherapy:In Press.

86. **Ellis, J. S., and D. W. G. Brown.** 1998. PCR for the detection of influenza viruses in clinical material, p. 119-127. *In* J. R. Stephenson and A. Warnes (ed.), Methods in Molecuiar Medicine, Vol 12: Diagnostic Viroiogy Protocols, vol. 12. Humana Press Inc, Totowa, NJ.

87. **Ellis, J. S., C. J. Sadler, P. Laidler, H. R. de Andrade, and M. C. Zambon.** 1997. Analysis of influenza A H3N2 strains isolated in England during 1995-1996 using polymerase chain reaction restriction. Journal of Medical Virology. **51:**234-241.

88. **Ellis, J. S., and M. C. Zambon.** 2001. A combined PCR - heteroduplex mobility assay for the detection and differentiation of influenza A viruses from different animal species. :In Press.

89. **Elton, D., L. Medcalf, K. Bishop, D. Harrison, and P. Digard.** 1999. Identification of amino acid residues of influenza virus nucleoprotein essential for RNA binding. Journal of Virology. **73:**7357-7367.

90. **Englund, J. A., R. E. Champlin, P. R. Wyde, H. Kantarjian, R. L. Atmar, J. Tarrand, H. Yousuf, H. Regnery, A. I. Klimov, N. J. Cox, and E. Whimbey.** 1998. Common emergence of amantadine- and rimantadine-resistant influenza A viruses in symptomatic immunocompromised adults. Clinical Infectious Diseases. **26:**1418-1424.

91. **Englund, J. A., C. J. Sullivan, M. C. Jordan, L. P. Dehner, G. M. Vercellotti, and H. H. Balfour.** 1988. Respiratory syncytial virus infection in immunocompromised adults. Annals of Internal Medicine. **109:**203-208.

92. **Enomoto, N., M. Kurosaki, Y. Tanaka, F. Marumo, and C. Sato.** 1994. Fluctuation of hepatitis C virus quasispecies in persistent infection and interferon treatment by single-strand conformation polymorphism analysis. Journal of General Virology. **75:**1361-1369.

93. **Evans, K. D., and M. W. Kline.** 1995. Prolonged influenza A infection responsive to rimantadine therapy in a human immunodeficiency virus-infected child. Pediatric Infectious Disease Journai. **14:**332-334.

94. **Ewart, G. D., T. Sutherland, P. W. Gage, and G. B. Cox.** 1996. The Vpu protein of human immunodeficiency virus type 1 forms cation-selective ion channels. Journal of Virology. **70:**7108-7115.

95. **Feldman, S., R. G. Webster, and M. Sugg.** 1977. Influenza in children and young adults with cancer: 20 cases. Cancer. **39:**350-353.

96. **Fitch, W. M., R. M. Bush, C. A. Bender, and N. J. Cox.** 1997. Long term trends in the evolution of H(3) HA1 human influenza type A. Proceedings of the National Academy of Sciences USA. **94:**7712-8.

97. **Fleming, D. M., P. Chakraverty, C. Sadler, and P. Litton.** 1995. Combined clinical and virological surveillance of influenza in winters of 1992 and 1993-4. British Medical Journal. **311:**290-291.

98. **Fleming, D. M., M. Zambon, A. I. Bartelds, and J. C. de Jong.** 1999. The duration and magnitude of influenza epidemics: a study of surveillance data from sentinel general practices in England, Wales and the Netherlands. European Journal of Epidemiology. **15:**467-473.

99. **Forrest, L. R., A. Kubol, I. T. Arkin, D. P. Tieleman, and S. P. Sansom.** 2000. Exploring models of the influenza M2 channel: MD simulation in a phosphoiipid bilayer. Biophysical Journal. **78:**55-69.

100. **Frace, A. M., A. I. Klimov, T. Rowe, R. A. Black, and J. M. Katz.** 1999. Modified M2 proteins produce heterotypic immunity against influenza A virus. Vaccine. **17:**2237-2244.

101. **Francis, T.** 1953. Vaccination against influenza. Bulletin of the World Health Organisation. **8:**725-741.

102. **Francis, T., and T. P. Magill.** 1936. The incidence of neutralising antibodies for human influenza virus in the serum of human individuals of different ages. Journal of Experimental Medicine. **63:**655-668.

103. **Frielle, D. W., D. D. Huang, and J. S. Youngner.** 1984. Persistent infection with influenza A virus: evolution of virus mutants. Virology. **138:**103-117.

104. **Garcia-Sastre, A., A. Egorov, D. Matassov, S. Brandt, D. E. Levy, J. E. Durbin, P. Palese, and T. Muster.** 1998. Influenza A virus lacking the NS1 gene repiicates in interferon-deficient systems. Virology. **252:**324-330.

105. **Garcia-Sastre, A., N. Percy, W. Barclay, and P. Palese.** 1994. Introduction of foreign sequences into the genome of influenza A virus. Developments in Biological Standardisation. **82:**237-246.

106. **Gaudin, Y., R. W. H. Ruigrok, and J. Brunner.** 1995. Low-pH induced conformational changes in virai fusion proteins: implications for the fusion mechanism. Journal of General Virology. **76:**1541-1556.

107. **Genovese, D., S. Catone, M. E. Farah, A. Gambacorta, and L. Fiore.** 1999. Isolation and biological characterisation of 3(2H)-isoflavene-resistant and -dependent poliovirus type 2 Sabin mutants. Journal of General Virology. **80:**157-167.

108. **Glezen, W. P., L. H. Taber, A. L. Frank, W. C. Gruber, and P. A. Piedra.** 1997. Influenza virus infections in infants. Pediatric Infectious Disease Journal. **16:**1065-1068.

109. **Glück, R., R. Mischler, B. Finkel, J. U. Que, B. Scarpa, and S. J. Cryz.** 1994. Immunogenicity of new virosome influenza vaccine in elderly people. Lancet. **344:**160-163.

110. **Gojobori, T., E. N. Moriyama, and M. Kimura.** 1990. Molecular clock of viral evolution, and the neutral theory. Proceedings of the National Academy of Sciences USA. **87:**10015-10018.

111. **Gorman, O. T., W. J. Bean, Y. Kawaoka, I. Donatelli, Y. J. Guo, and R. G. Webster.** 1991. Evolution of influenza A virus nucleoprotein genes: implications for the origins of H1N1 human and classical swine viruses. Journal of Virology. **65:**3704-3714.

112. **Gorman, O. T., W. J. Bean, Y. Kawaoka, and R. G. Webster.** 1990. Evolution of the nucleoprotein gene of influenza A virus. Journal of Virology. **64:**1487-1497.

113. **Gorman, O. T., W. J. Bean, and R. G. Webster.** 1992. Evolutionary processes in influenza viruses: divergence, rapid evolution, and stasis. Current Topics in Microbiology and Immunology. **176:**75-97.

114. **Gorman, O. T., R. O. Donis, Y. Kawaoka, and R. G. Webster.** 1990. Evolution of influenza A virus PB2 genes: implications for evolution of the ribonucleoprotein complex and origin of human influenza A virus. Journal of Virology. **64:**4893-4902.

115. **Goshima, F. Y., and K. Maeno.** 1989. Persistent infection of MDCK cells by influenza C virus: initiation and characterisation. Journal of General Virology. **70:**3481-3485.

116. **Gotch, F., A. McMichael, G. Smith, and B. Moss.** 1987. Identification of viral molecules recognized by influenza-specific human cytotoxic T lymphocytes. Journal of Experimental Medicine. **165:**408-416.

117. **Gottlieb, T., and D. Mitchell.** 1998. The independent evolution of resistance to ciprofloxacin, rifampicin, and fusidic acid in methicillin-

resistant Staphylococcus aureus in Australian teaching hospitals (1990-1995). Journal of Antimicrobial Chemotherapy. **42:**67-73.

118. **Grambas, S., M. S. Bennett, and A. J. Hay.** 1992. Influence of amantadine-resistance mutations on the pH regulatory function of the M2 protein of influenza A viruses. Virology. **191:**541-549.

119. **Gravenstein, S., P. Drlnka, D. Osterwell, M. Schilling, J. McElhaney, M. Elliott, J. Hammond, O. Keene, P. Krause, and N. Flack.** 2000. A multicenter prospective double-blind randomised controlled trial comparing relative safety and efficacy of zanamivir to rimantadine for nursing hone influenza outbreak control. 40th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), Toronto, Canada.

120. **Gregoriadis, G., L. Tan, E. T. S. Ben-Ahmeida, and R. Jennings.** 1992. Liposomes enhance the immunogenicity of reconstituted influenza virus A/PR/8 envelopes and the formation of protective antibody by influenza virus A/Sichuan/87 (H3N2) surface antigen. Vaccine. **10:**747-753.

121. **Grist, N. R.** 1979. Pandemic influenza 1918. British Medical Journal:1632-1633.

122. **Grist, N. R., C. A. Ross, and E. J. Bell.** 1974. , p. 39-45, Diagnostic Methods in Clinical Virology 2nd Ed. Blackwell Scientific Publications, Oxford.

123. **Gross, P. A., H. Lee, J. A. Wolff, C. B. Hall, A. B. Minnefore, and M. E. Lazicki.** 1978. Influenza immunisation in immunosuppressed children. Journal of Pediatrics. **92:**30-35.

124. **Gubareva, L. V., R. Bethell, G. J. Hart, K. G. Murti, C. R. Penn, and R. G. Webster.** 1996. Characterisation of mutants of influenza A virus selected with the neuraminidase inhibitor 4-guanidino-Neu5Ac2en. Journal of Virology. **70:**1818-1827.

125. **Gubareva, L. V., L. Kaiser, and F. G. Hayden.** 2000. Influenza virus neuraminidase inhibitors. Lancet. **355:**827-835.

126. **Gubareva, L. V., L. Kaiser, M. N. Matrosovich, Y. Soo-Hoo, and F. G. Hayden.** 2001. Selection of influenza virus mutants in experimentally infected volunteers treated with oseltamivir. Journal of Infectious Diseases. **183:**523-531.

127. **Gubareva, L. V., M. N. Matrosovich, M. K. Brenner, R. C. Bethell, and R. G. Webster.** 1998. Evidence for zanamivir resistance in an

immunocompromised child infected with influenza B virus. Journal of Infectious Diseases. **178:**1257-1262.

128. **Gubareva, L. V., M. J. Robinson, R. C. Betheii, and R. G. Webster.** 1997. Catalytic and framework mutations in the neuraminidase active site of influenza viruses that are resistant to 4-guanidino-Neu5Ac2en. Journal of Virology. **71:**3385-90.

129. **Guo, Y., J. Li, X. Cheng, M. Wang, Y. Zhou, X. H. Li, F. Cai, H. L. Miao, H. Zhang, and F. Guo.** 1999. Discovery of men infected by an avian influenza A (H9N2) virus. Chinese Journal of Experimental and Clinical Virology. **13:**105-108.

130. **Hall, C. B., R. Dolin, C. L. Gala, D. M. Markovitz, Y. Q. Zhang, P. H. Madore, F. A. Disney, W. B. Talpey, J. L. Green, A. B. Francis, and M. E. Pichichero.** 1987. Children with influenza A infection: treatment with rimantadine. Pediatrics. **80:**275-282.

131. **Hall, C. B., E. E. Waish, J. F. Hruska, R. F. Betts, and W. J. Hall.** 1983. Ribavirin treatment of experimental respiratory syncytial viral infection. A controlled double-blind study in young adults. Journal of the American Medical Association. **249:**2666-2670.

132. **Hamzawi, M., R. Jennings, and C. W. Potter.** 1981. The amantadine-sensitivity of recombinant and parental influenza virus strains. Medical Microbiology and Immunology. **169:**259-268.

133. **Hankins, R. W., K. Nagata, A. Kato, and A. Ishihama.** 1990. Mechanism of influenza virus transcription inhibition by matrix (M1) protein. Research in Virology. **141:**305-314.

134. **Harrison, C., L. Jenski, N. Cox, M. Gilchrist, S. Alter, T. Sketch, M. Harmon, R. Harris, and A. Kendal.** 1986. Onset of cell-mediated immune function after bone marrow transplantation coincides with cessation of chronic shedding of influenza A virus, p. 487-496, Options for the Control of Influenza II. Alan R. Liss, Inc.

135. **Hausmann, J., E. Kretzschmar, W. Garten, and H. D. Klenk.** 1995. N1 neuraminidase of influenza virus A/FPV/Rostock/34 has haemadsorbing activity. Journal of General Virology. **76:**1719-1728.

136. **Hay, A. J.** 1992. The action of adamantanamines against influenza A viruses: inhibition of the M2 ion channel protein. Seminars in Virology. **3:**21-30.

137. **Hay, A. J.** 1996. Amantadine and rimantadine: mechanisms, p. 43-58. *In* D. D. Richman (ed.), Antiviral Drug-Resistance. John Wiley & Sons Ltd.

138. **Hay, A. J.** 1998. Functional properties of the virus ion channels, p. 74-81. *In* K. G. Nicholson and R. G. Webster and A. J. Hay (ed.), Textbook of Influenza. Blackwell Science, Oxford.

139. **Hay, A. J.** 1998. The virus genome and its replication, p. 43-53. *In* K. G. Nicholson and R. G. Webster and A. J. Hay (ed.), Textbook of Influenza. Blackwell Science, Oxford.

140. **Hay, A. J., N. C. Kennedy, J. J. Skehel, and G. Appleyard.** 1979. The matrix protein gene determines amantadine-sensitivity of influenza viruses. Journal of General Virology. **42:**189-191.

141. **Hay, A. J., J. J. Skehel, and J. McCauley.** 1982. Characterisation of influenza virus RNA complete transcripts. Virology. **116:**517-522.

142. **Hay, A. J., A. J. Wolstenholme, J. J. Skehel, and M. H. Smith.** 1985. The molecular basis of the specific anti-influenza action of amantadine. EMBO Journal. **4:**3021-3024.

143. **Hay, A. J., and M. C. Zambon.** 1984. Multiple actions of amantadine against influenza viruses, p. 301-315. *In* Y. Becker (ed.), Antiviral Drugs and Interferon: the molecular basis of their activity. Martinus Nijhoff Publishing, Boston.

144. **Hayden, F. G.** 1996. Amantadine and rimantadine: clinical aspects, p. 59-77. *In* D. D. Richman (ed.), Antiviral Drug-Resistance. John Wiley & Sons Ltd.

145. **Hayden, F. G.** 1997. Prevention and treatment of influenza in immunocompromised patients. American Journal of Medicine. **102:**55-60.

146. **Hayden, F. G., R. L. Atmar, M. Schilling, C. Johnston, D. Poretz, D. Paar, L. Huson, P. Ward, and R. G. Mills.** 1999. Use of the selective oral neuraminidase inhibitor oseltamivir to prevent influenza. New England Journal of Medicine. **341:**1336-1343.

147. **Hayden, F. G., R. B. Belshe, R. D. Clover, A. J. Hay, M. G. Oakes, and W. Soo.** 1989. Emergence and apparent transmission of rimantadine-resistant influenza A virus in families. New England Journal of Medicine. **321:**1696-1702.

148. **Hayden, F. G., K. M. Cote, and R. G. Douglas, Jr.** 1980. Plaque inhibition assay for drug susceptibility testing of influenza viruses. Antimicrobial Agents and Chemotherapy. **17:**865-870.

149. **Hayden, F. G., and A. S. Monto.** 1986. Oral rimantadine hydrochloride therapy of influenza A virus H3N2 subtype infection in adults. Antimicrobial Agents and Chemotherapy. **29:**339-341.

150. **Hayden, F. G., A. D. Osterhaus, J. J. Treanor, D. M. Fleming, F. Y. Aoki, K. G. Nicholson, A. M. Bohnen, H. M. Hirst, O. Keene, and K. Wightman.** 1997. Efficacy and safety of the neuraminidase inhibitor zanamivir in the treatment of influenza virus infections. New England Journal of Medicine. **337:**874-880.

151. **Hayden, F. G., C. A. Sable, J. D. Connor, and J. Lane.** 1996. Intravenous ribavirin by constant infusion for serious influenza and parainfluenza infection. Antiviral Therapy. **1:**51-56.

152. **Hayden, F. G., S. J. Sperber, R. B. Belshe, R. D. Clover, A. J. Hay, and S. Pyke.** 1991. Recovery of drug-resistant influenza A virus during therapeutic use of rimantadine. Antimicrobial Agents and Chemotherapy. **35:**1741-1747.

153. **Hayden, F. G., J. J. Treanor, R. F. Betts, M. Lobo, J. D. Esinhart, and E. K. Hussey.** 1996. Safety and efficacy of the neuraminidase inhibitor GG167 in experimental human influenza. Journal of the American Medical Association. **275:**295-299.

154. **Hayden, F. G., J. J. Treanor, R. S. Fritz, M. Lobo, R. F. Betts, M. Miller, N. Kinnersley, R. G. Mills, P. Ward, and S. E. Straus.** 1999. Use of the oral neuraminidase inhibitor oseltamivir in experimental human influenza: randomised controlled trials for prevention and treatment. Journal of the American Medical Association. **282:**1240-1246.

155. **Hecht, F. M., R. M. Grant, C. J. Petropoulos, B. Dillon, M. A. Chesney, H. Tian, N. S. Hellmann, N. I. Bandrapalli, L. Digllio, B. Branson, and J. O. Kahn.** 1998. Sexual transmission of an HIV-1 variant resistant to multiple reverse-transcriptase and protease inhibitors. New England Journal of Medicine. **339:**307-311.

156. **Helder, H., B. Adamczyk, H. W. Presber, C. Schroeder, R. Feldblum, and M. K. Indulen.** 1981. Occurrence of amantadine- and rimantadine-resistant influenza A virus strains during the 1980 epidemic. Acta Virologica. **25:**395-400.

157. **Henkel, J. R., and O. A. Weisz.** 1998. Influenza virus M2 protein slows traffic along the secretory pathway; pH perturbation of acidified

compartments affects early golgi transport steps. The Journal of Biological Chemistry. **273:**6518-6524.

158. **Hirst, G. K.** 1942. Adsorption of influenza heamagglutinins and virus by red blood cells. Journal of Experimental Medicine. **76:**195-209.

159. **Holland, J., K. Spindler, F. Horodyski, E. Grabau, S. Nichol, and S. Van de Pol.** 1982. Rapid evolution of RNA genomes. Science. **215:**1577-1585.

160. **Holland, J. J., J. C. De La Torre, and D. A. Steinhauer.** 1992. RNA virus populations as quasispecies. Current Topics in Microbiology and Immunology. **176:**1-20.

161. **Holsinger, L. J., and R. A. Lamb.** 1991. Influenza virus M2 integral membrane protein is a homotetramer stabilized by formation of disulfide bonds. Virology. **183:**32-43.

162. **Holsinger, L. J., M. A. Shaughnessy, A. Micko, L. H. Pinto, and R. A. Lamb.** 1995. Analysis of the posttranslational modifications of the influenza virus M2 protein. Journal of Virology. **69:**1219-1225.

163. **Hongo, S., K. Sugawara, Y. Muraki, F. Kitame, and K. Nakamura.** 1997. Characterisation of a second protein (CM2) encoded by RNA segment 6 of influenza C virus. Journal of Virology. **71:**2786-2792.

164. **Hongo, S., K. Sugawara, H. Nishimura, Y. Muraki, F. Kitame, and K. Nakamura.** 1994. Identification of a second protein encoded by influenza C virus RNA segment 6. Journal of General Virology. **75:**3503-3510.

165. **Hornick, R. B., Y. Togo, S. Mahler, and D. Iezzoni.** 1969. Evaluation of amantadine hydrochloride in the treatment of A2 influenzal disease. Bulletin of the World Health Organisation. **41:**671-676.

166. **Houck, P., M. Hemphill, S. LaCroix, D. Hirsh, and N. Cox.** 1995. Amantadine-resistant influenza A in nursing homes: identification of a resistant virus prior to drug use. Archives Internal Medicine. **155:**533-537.

167. **Hruska, J. F., J. M. Bernstein, R. G. Douglas, and C. B. Hall.** 1980. Effects of ribavirin on respiratory syncytial virus *in vitro*. Antimicrobial Agents and Chemotherapy. **17:**770-775.

168. **Hull, J. D., R. Gilmore, and R. A. Lamb.** 1988. Integration of a small integral membrane protein, M2, of influenza virus into the endoplasmic reticulum: analysis of the internal signal-anchor domain of a protein with an ectoplasmic NH2 terminus. Journal of Cell Biology. **106:**1489-1498.

169. **Hutchinson, E. J., C. A. Joseph, P. Chakraverty, M. Zambon, D. M. Fleming, and J. M. Watson.** 1995. Influenza surveillance in England and

Wales: October 1994 to June 1995. Communicable Disease Report Review. **5:**R200-204.

170. **Hutchinson, E. J., C. A. Joseph, M. Zambon, D. M. Fleming, and J. M. Watson.** 1996. Influenza surveillance in England and Wales: October 1995 to June 1996. Communicable Disease Report Review. **6:**R163-169.

171. **Ikeda, S., J. Neyts, S. Verma, A. Wickramasinghe, P. Mohan, and E. de Clercq.** 1994. *In vitro* and *in vivo* inhibition of ortho- and paramymxovirus infections by a new class of sulfonic acid polymers interacting with virus-cell binding and/or fusion. Antimicrobial Agents and Chemotherapy. **38:**256-259.

172. **Imrie, A., A. Beveridge, W. Genn, J. Vizzard, and D. A. Cooper.** 1997. Transmission of human immunodeficiency virus type 1 resistant to nevirapine and zidovudine. Journal of Infectious Diseases. **175:**1502-1506.

173. **Ina, Y., and T. Gojobori.** 1994. Statistical analysis of nucleotide sequences of the hemagglutinin gene of human influenza A viruses. Proceedings of the National Academy of Sciences USA. **91:**8388-8392.

174. **Ishihama, A., and P. Barbier.** 1994. Molecular anatomy of viral RNA-directed RNA polymerases. Archives of Virology. **134:**235-258.

175. **Ito, T., O. T. Gorman, Y. Kawaoka, W. J. Bean, and R. G. Webster.** 1991. Evolutionary analysis of the influenza A virus M gene with comparison of the M1 and M2 proteins. Journal of Virology. **65:**5491-5498.

176. **Jameson, J., J. Cruz, and F. A. Ennis.** 1998. Human cytotoxic T-lymphocyte repertoire to influenza A viruses. Journal of Virology. **72:**8682-8689.

177. **Kaplan, M. M., and R. G. Webster.** 1977. The epidemiology of influenza. Scientific American. **237:**88-106.

178. **Kati, W., D. Montgomery, K. Steffy, R. Carrick, Y. Shi, C. Maring, K. McDaniel, V. Stoll, W. G. Laver, A. Molla, and W. Kohlbrenner.** 2001. *In vitro* characterisation of ABT-675: a potent, broad spectrum inhibitor of influenza. The Origin and Control of Pandemic Influenza. The Royal Society, London.

179. **Kato, N., and H. J. Eggers.** 1969. Inhibition of uncoating of fowl plague virus by l-adamantanamine hydrochloride. Virology. **37:**632-641.

180. **Kawaoka, Y., O. T. Gorman, T. Ito, K. Wells, R. O. Donis, M. R. Castrucci, I. Donatelli, and R. G. Webster.** 1998. Influence of host

species on the evolution of the nonstructural (NS) gene of influenza A viruses. Virus Research. **55:**143-156.

181. **Kawaoka, Y., S. Krauss, and R. G. Webster.** 1989. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. Journal of Virology. **63:**4603-4608.

182. **Kawaoka, Y., and R. G. Webster.** 1988. Sequence requirements for cleavage activation of influenza virus haemagglutinin expressed in mammalian cells. Proceedings of the National Academy of Sciences USA. **85:**324-328.

183. **Kempe, A., C. B. Hall, N. E. MacDonald, H. R. Foye, K. A. Woodin, H. J. Cohen, E. D. Lewis, M. Gullace, C. L. Gala, C. S. Dulberg, and et al.** 1989. Influenza in children with cancer. Journal of Pediatrics. **115:**33-39.

184. **Kendal, A. P., and H. D. Klenk.** 1991. Amantadine inhibits an early, M2 protein-dependent event in the replication cycle of avian influenza (H7) viruses. Archives of Virology. **119:**265-73.

185. **Kendal, A. P., H. F. Maassab, G. I. Alexandrova, and Y. Z. Ghendon.** 1981. Development of cold-adapted recombinant live, attenuated influenza A vaccines in the U.S.A. and U.S.S.R. Antiviral Research. **1:**339-365.

186. **Kilbourne, E. D.** 1977. Influenza pandemics in perspective. Journal of the American Medical Association. **237:**1225-1228.

187. **Kilbourne, E. D.** 1987. Replication of influenza viruses, p. 57-87, Influenza. Plenum Medical Book Company, New York.

188. **Kilbourne, E. D., C. P. Cerini, M. W. Khan, J. W. Mitchell, Jr., and P. L. Ogra.** 1987. Immunologic response to the influenza virus neuraminidase is influenced by prior experience with the associated viral haemagglutinin. I. Studies in human vaccinees. Journal of Immunology. **138:**3010-3013.

189. **Kilbourne, E. D., W. G. Laver, J. L. Schulman, and R. G. Webster.** 1968. Antiviral activity of antiserum specific for an influenza virus neuraminidase. Journal of Virology. **2:**281-288.

190. **Kim, C. U., W. Lew, M. A. Williams, H. Liu, L. Zhang, S. Swaminathan, N. Bischofberger, M. S. Chen, D. B. Mendel, C. Y. Tai, W. G. Laver, and R. C. Stevens.** 1997. Influenza neuraminidase inhibitors possessing a novel hydrophobic interaction in the enzyme active site: design, synthesis and structural analysis of carbocyclic sialic acid analogues with potent anti-influenza activity. Journal of the American Chemical Society. **119:**681-690.

191. **Klenk, H. D., and R. Rott.** 1988. The molecular biology of influenza virus pathogenicity. Advances in Virus Research. **34:**247-281.

192. **Klenk, H. D., R. Rott, M. Orlich, and J. Blödorn.** 1975. Activation of influenza A viruses by trypsin treatment. Virology. **68:**426-439.

193. **Klimov, A. I., E. Rocha, F. G. Hayden, P. A. Shult, L. F. Roumillat, and N. J. Cox.** 1995. Prolonged shedding of amantadine-resistant influenza A viruses by immunodeficient patients: detection by polymerase chain reaction-restriction analysis. Journal of Infectious Diseases. **172:**1352-1355.

194. **Kobayashi, M., T. Toyoda, and A. Ishihama.** 1996. Influenza virus PB1 protein is the minimal and essential subunit of RNA polymerase. Archives of Virology. **141:**525-539.

195. **Krug, R. M., F. V. Aloso-Caplen, I. Julkunon, and M. G. Katze.** 1989. Expression and replication of the influenza virus genome, p. 89-152. *In* R. M. Krug (ed.), The Influenza Viruses. Plenum Press, New York.

196. **Kubar, O. I., E. A. Brjantseva, L. E. Nikitina, and D. M. Zlydnikov.** 1989. The importance of virus drug-resistance in the treatment of influenza with rimantadine. Antiviral Research. **11:**313-315.

197. **Kung, H. C., K. F. Jen, W. C. Yuan, S. F. Tien, and C. M. Chu.** 1978. Influenza in China in 1977: recurrence of influenza virus A subtype H1N1. Bulletin of the World Health Organisation. **56:**913-918.

198. **Kurtz, S., G. Luo, K. M. Hahnenberger, C. Brooks, O. Gecha, K. Ingalls, K. Numata, and M. Krystal.** 1995. Growth impairment resulting from expression of influenza virus M2 protein in *Saccharomyces cerevisiae*: identification of a novel inhibitor of influenza virus. Antimicrobial Agents and Chemotherapy. **39:**2204-2209.

199. **Lamb, R. A.** 1989. Genes and proteins of the influenza viruses, p. 1-87. *In* R. M. Krug (ed.), The Influenza Viruses. Plenum Press, New York.

200. **Lamb, R. A., and P. W. Choppin.** 1981. Identification of a second protein (M2) encoded by RNA segment 7 of influenza virus. Virology. **112:**729-737.

201. **Lamb, R. A., P. W. Choppin, R. M. Chanock, and C. J. Lai.** 1980. Mapping of the two overlapping genes for polypeptides NS1 and NS2 on RNA segment 8 of influenza virus genome. Proceedings of the National Academy of Sciences USA. **77:**1857-1861.

202. **Lamb, R. A., L. J. Holsinger, and L. H. Pinto.** 1994. The influenza A virus M2 ion channel protein and its role in the influenza virus life cycle, p. 303-

321. *In* E. Wimmer (ed.), Cellular Receptors for Animal Viruses. CSHL Press.

203. **Lamb, R. A., and C. J. Lai.** 1981. Conservation of the influenza virus membrane protein (M1) amino acid sequence and an open reading frame of RNA segment 7 encoding a second protein (M2) in H1N1 and H3N2 strains. Virology. **112:**746-751.

204. **Lamb, R. A., C.-J. Lai, and P. W. Choppin.** 1981. Sequences of mRNAs derived from genome RNA segment 7 of influenza virus: colinear and interrupted mRNAs code for overlapping proteins. Proceedings of the National Academy of Sciences USA. **78:**4170-4174.

205. **Lamb, R. A., and L. H. Pinto.** 1997. Do Vpu and Vpr of human immunodeficiency virus type 1 and NB of influenza B virus have ion channel activities in the viral life cycles? Virology. **229:**1-11.

206. **Lamb, R. A., S. L. Zebedee, and C. D. Richardson.** 1985. Influenza virus M2 protein is an integral membrane protein expressed on the infected-cell surface. Cell. **40:**627-633.

207. **Lambkin, R., and J. S. Oxford.** 1998. Alternative influenza antiviral agents, p. 488-505. *In* K. G. Nicholson and R. G. Webster and A. J. Hay (ed.), Textbook of Influenza. Blackwell Sciences, Oxford.

208. **Lapatschek, M. S., M. Marschall, and H. Meier-Ewert.** 1995. The persistent variant of influenza C virus carries one characteristic point mutation in RNA segment 1. Virus Research. **39:**47-54.

209. **Laver, W. G., G. M. Air, R. G. Webster, and L. J. Markoff.** 1982. Amino acid sequence changes in antigenic variants of type A influenza virus N2 neuraminidase. Virology. **122:**450-460.

210. **Laver, W. G., N. Bischofberger, and R. G. Webster.** 1999. Disarming flu viruses. Scientific American. **280:**78-87.

211. **Lee, C., M. Loeb, A. Phillips, J. Nesbitt, K. Smith, M. Fearon, M. A. McArthur, T. Mazzulli, Y. Li, and A. McGeer.** 2000. Zanamivir use during transmission of amantadine-resistant influenza A in a nursing home. Infection Control and Hospital Epidemiology. **21:**700-4.

212. **Lee, C., M. Loeb, A. Phillips, J. Nesbitt, K. Smith, M. Fearon, M. A. McArthur, T. Mazzulli, Y. Li, and A. McGeer.** 2000. Zanamivir use during transmission of amantadine-resistant influenza A in a nursing home. Infection Control and Hospital Epidemiology. **21:**700-704.

213. **Lee, S. W., J. W. Youn, B. L. Seong, and Y. C. Sung.** 1999. IL-6 induces long-term protective immunity against a lethal challenge of influenza virus. Vaccine. **17:**490-496.

214. **Lees, W. J., A. Spaltenstein, J. E. Kingery-Wood, and G. M. Whitesides.** 1994. Polyacrylamides bearing pendant alpha-sialoside groups strongly inhibit agglutination of erythrocytes by influenza A virus: multivalency and steric stabilization of particulate biological systems. Journal of Medicinal Chemistry. **37:**3419-3433.

215. **Lewinsohn, D. M., R. A. Bowden, D. Mattson, and S. W. Crawford.** 1996. Phase I study of intravenous ribavirin treatment of respiratory syncytial virus pneumonia after marrow transplantation. Antimicrobial Agents and Chemotherapy. **40:**2555-2557.

216. **Lewis, V. A., R. Champlin, J. Englund, R. Couch, J. M. Goodrich, K. Rolston, D. Przepiorka, N. Q. Mirza, H. M. Yousuf, M. Luna, G. P. Bodey, and E. Whimbey.** 1996. Respiratory disease due to parainfluenza virus in adult bone marrow transplant recipients. Clinical Infectious Diseases. **23:**1033-1037.

217. **Li, W. H.** 1997. Molecular Evolution. Sinauer Associates, Inc., Sunderland, MA.

218. **LI, Z. N., S. Hongo, K. Sugawara, K. Sugahara, E. Tsuchiya, Y. Matsuzaki, and K. Nakamura.** 2001. The sites for fatty acylation, phosphorylation and intermolecular disulphide bond formation of influenza C virus CM2 protein. Journal of General Virology. **82:**1085-1093.

219. **Lin, J. C., and K. L. Nichoi.** 2001. Excess mortality due to pneumonia or influenza during influenza seasons among persons with acquired immunodeficiency syndrome. Archives of International Medicine. **161:**441-446.

220. **Lindstrom, S., A. Endo, S. Sugita, M. Pecoraro, Y. Hiromoto, M. Kamada, T. Takahashi, and K. Nerome.** 1998. Phylogenetic analyses of the matrix and non-structural genes of equine influenza viruses. Archives of Virology. **143:**1585-1598.

221. **Little, S. J., E. S. Daar, R. T. D'Aquila, P. H. Keiser, E. Connick, J. M. Whitcomb, N. S. Helimann, C. J. Petropoulos, L. Sutton, J. A. Pitt, E. S. Rosenberg, R. A. Koup, B. D. Walker, and D. D. Richman.** 1999. Reduced antiretroviral drug susceptibility among patients with primary HIV infection. Journal of the American Medical Association. **282:**1142-1149.

222. **Liu, C., M. C. Eicheiberger, R. W. Compans, and G. M. Air.** 1995. Influenza type A virus neuraminidase does not play a role in viral entry, replication, assembly, or budding. Journal of Virology. **69:**1099-1106.

223. **Ljungman, P., J. Andersson, J. Aschan, L. Barkhoit, A. Ehrnst, M. Johansson, and O. Weiland.** 1993. Influenza A in immunocompromised patients. Clinical Infectious Diseases. **17:**244-247.

224. **Lubeck, M. D., J. L. Schulman, and P. Palese.** 1978. Susceptibility of influenza A viruses to amantadine is influenced by the gene coding for M protein. Journal of Virology. **28:**710-716.

225. **Lucas, W. T., P. Whitaker-Dowling, C. R. Kaifer, and J. S. Youngner.** 1988. Characterisation of a unique protein produced by influenza A virus recovered from a long-term persistent infection. Virology. **166:**620-623.

226. **Ludwig, S., U. Schultz, J. Mandler, W. M. Fitch, and C. Scholtissek.** 1991. Phylogenetic relationship of the nonstructural (NS) genes of influenza A viruses. Virology. **183:**566-577.

227. **Lundberg, K. S., D. D. Shoemaker, M. W. Adams, J. M. Short, J. A. Sorge, and E. J. Mathur.** 1991. High-fidelity amplification using a thermostable DNA polymerase isolated from *Pyrococcus furiosus*. Gene. **108:**1-6.

228. **M.I.S.T.** 1998. Randomised trial of efficacy and safety of inhaled zanamivir in treatment of influenza A and B virus infections. Lancet. **352:**1877-1881.

229. **Magnussen, C. R., J. Douglas, R.G., R. F. Betts, F. K. Roth, and M. P. Meagher.** 1977. Double-blind evaluation of oral ribavirin (virazole) in experimental influenza A virus infection in volunteers. Antimicrobial Agents and Chemotherapy. **12:**498-502.

230. **Mäkelä, M. J., K. Pauksens, T. Rostiie, D. M. Flemming, C. Y. Man, O. N. Keene, and A. Webster.** 2000. Clinical efficacy and safety of the orally inhaled neuraminidase inhibitor zanamivir in the treatment of influenza: a randomized, double-blind, placebo-controlled European study. Journal of Infection. **40:**42-48.

231. **Mammen, M., G. Dahmann, and G. M. Whitesides.** 1995. Effective inhibitors of hemagglutination by influenza virus synthesized from polymers having active ester groups. Insight into mechanism of inhibition. Journal of Medicinal Chemistry. **38:**4179-4190.

232. **Marion, R. M., T. Aragon, A. Beloso, A. Nieto, and J. Ortin.** 1997. The N-terminal half of the influenza virus NS1 protein is sufficient for nuclear

retention of mRNA and enhancement of viral mRNA translation. Nucleic Acids Research. **25:**4271-4277.

233. **Markushin, S., H. Ghiasi, N. Sokolov, A. Shilov, B. Sinitsin, D. Brown, A. Klimov, and D. Nayak.** 1988. Nucleotide sequence of RNA segment 7 and the predicted amino sequence of M1 and M2 proteins of FPV/Weybridge (H7N7) and WSN (H1N1) influenza viruses. Virus Research. **10:**263-271.

234. **Marschall, M., A. Heiten, A. Hechtfischer, A. Zach, and H. Meier-Ewert.** 1998. Persistent infection with an influenza C virus variant is dominantly established in the presence of the parental wild-type virus. Virus Research. **54:**51-58.

235. **Marsh, M., and A. Helenius.** 1989. Virus entry into animal cells. Advances in Virus Research. **36:**107-151.

236. **Martin, J., S. A. Wharton, Y. P. Lin, D. K. Takemoto, J. J. Skehel, D. C. Wiley, and D. A. Steinhauer.** 1998. Studies of the binding properties of influenza haemagglutinin receptor-site mutants. Virology. **241:**101-111.

237. **Martinez, C., L. del Rio, A. Portela, E. Domingo, and J. Ortin.** 1983. Evolution of the influenza virus neuraminidase gene during drift of the N2 subtype. Virology. **130:**539-545.

238. **Mast, E. E., M. W. Harmon, S. Gravenstein, S. P. Wu, N. H. Arden, R. Circo, G. Tyszka, A. P. Kendal, and J. P. Davis.** 1991. Emergence and possible transmission of amantadine-resistant viruses during nursing home outbreaks of influenza A (H3N2). American Journal of Epidemiology. **134:**988-997.

239. **Mauch, T. J., S. Bratton, T. Myers, E. Krane, S. R. Gentry, and C. E. Kashtan.** 1994. Influenza B virus infection in paediatric solid organ transplant recipients. Pediatrics. **94:**225-229.

240. **McCammon, J. R., and V. W. Riesser.** 1979. Effects of ribavirin on BHK-21 cells acutely or persistently infected with mumps virus. Antimicrobial Agents and Chemotherapy. **15:**356-360.

241. **McCullers, J. A., S. Facchini, P. J. Chesney, and R. G. Webster.** 1999. Influenza B virus encephalitis. Clinical Infectious Diseases. **28:**898-900.

242. **McKimm-Breschkin, J. L., T. J. Blick, A. Sahasrabudhe, T. Tiong, D. Marshall, G. J. Hart, R. C. Betheli, and C. R. Penn.** 1996. Generation and characterisation of variants of NWS/G70C influenza virus after *in vitro*

passage in 4-amino-Neu5Ac2en and 4-guanidino-Neu5Ac2en. Antimicrobial Agents and Chemotherapy. **40:**40-46.

243. **McKimm-Breschkin, J. L., A. Sahasrabudhe, T. J. Biick, M. McDonald, P. M. Coiman, G. J. Hart, R. C. Betheii, and J. N. Varghese.** 1998. Mutations in a conserved residue in the influenza virus neuraminidase active site decreases sensitivity to Neu5Ac2en-derived inhibitors. Journal of Virology. **72:**2456-2462.

244. **McMinn, P., A. Carreilo, C. Cole, D. Baker, and A. Hampson.** 1999. Antigenic drift of influenza A (H3N2) virus in a persistently infected immunocompromised host is similar to that occurring in the community. Clinical Infectious Diseases. **29:**456-458.

245. **Mendel, D. B., C. Y. Tai, P. A. Escarpe, W. Li, R. W. Sidweli, J. H. Huffman, C. Sweet, K. J. Jakeman, J. Merson, S. A. Lacy, W. Lew, M. A. Williams, L. Zhang, M. S. Chen, N. Bischofberger, and C. U. Kim.** 1998. Oral administration of a prodrug of the influenza virus neuraminidase inhibitor GS 4071 protects mice and ferrets against influenza infection. Antimicrobial Agents and Chemotherapy. **42:**640-646.

246. **Meyer, B. J., and C. S. Schmaijohn.** 2000. Persistent hantavirus infections: characteristics and mechanisms. Trends in Microbiology. **8:**61-67.

247. **Mitnaui, L. J., M. R. Castrucci, K. G. Murti, and Y. Kawaoka.** 1996. The cytoplasmic tail of influenza A virus neuraminidase (NA) affects NA incorporation into virions, virion morphology, and virulence in mice but is not essential for virus replication. Journal of Virology. **70:**873-879.

248. **Moneret-Vautrin, D. A., and J. P. Griiliat.** 1977. Influenza vaccine and egg allergy. Lancet. **??:**??

249. **Monto, A. S.** 1998. Guidelines for the clinical use of antivirals, p. 506-514. *In* K. G. Nicholson and R. G. Webster and A. J. Hay (ed.), Textbook of Influenza. Blackwell Science Ltd, Oxford.

250. **Monto, A. S., D. M. Flemming, D. Henry, R. de Groot, M. Makela, T. Klein, M. Elliott, O. N. Keene, and C. Y. Man.** 1999. Efficacy and safety of the neuraminidase inhibitor zanamivir in the treatment of influenza A and B virus infections. Journal of Infectious Diseases. **180:**254-261.

251. **Monto, A. S., R. A. Gunn, M. G. Bandyk, and C. L. King.** 1979. Prevention of Russian influenza by amantadine. Journal of the American Medical Association. **241:**1003-1007.

252. **Monto, A. S., D. P. Robinson, M. L. Herlocher, J. M. Hinson, M. J. Elliott, and A. Crisp.** 1999. Zanamivir in the prevention of influenza among healthy adults: a randomised controlled trial. Journal of the American Medical Association. **282:**31-35.

253. **Mould, J. A., H. C. Li, C. S. Dudlak, J. D. Lear, A. Pekosz, R. A. Lamb, and L. H. Pinto.** 2000. Mechanism for proton conduction of the M2 ion channel of influenza A virus. Journal of Biological Chemistry. **275:**8592-8599.

254. **Murphy, B. R.** 1993. Use of live attenuated cold-adapted influenza A reassortant virus vaccines in infants, children, young adults and elderly adults. Infection and Disease in Clinical Practice. **2:**174-181.

255. **Murphy, M. F.** 1978. *In vitro* inhibition of subacute sclerosing panencephalitis virus by the antiviral agent ribavirin. Journal of Infectious Diseases. **138:**249-251.

256. **Myc, A., M. J. Anderson, and J. R. Baker.** 1999. Optimisation of *in situ* cellular ELISA performed on influenza A virus-infected monolayers for screening of antiviral agents. Journal of Virological Methods. **77:**165-177.

257. **Nemeroff, M. E., S. M. Barabino, Y. Li, W. Keller, and R. M. Krug.** 1998. Influenza virus NS1 protein interacts with the cellular 30 kDa subunit of CPSF and inhibits 3'end formation of cellular pre-mRNAs. Molecular Cell. **1:**991-1000.

258. **Nichol, K. L., P. M. Mendelman, K. P. Mallon, L. A. Jackson, G. J. Gorse, R. B. Belshe, W. P. Glezen, and J. Wittes.** 1999. Effectiveness of live, attenuated intranasal influenza virus vaccine in healthy, working adults: a randomized controlled trial. Journal of the American Medical Association. **282:**137-144.

259. **Nicholson, K. G.** 1992. Clinical features of influenza. Seminars in Respiratory Infections. **7:**26-37.

260. **Nicholson, K. G.** 1998. Human influenza, p. 219-264. *In* K. G. Nicholson and R. G. Webster and A. J. Hay (ed.), Textbook of Influenza. Blackwell Science Ltd, Oxford.

261. **Nicholson, K. G., F. Y. Aoki, A. D. M. E. Osterhaus, S. Trottier, O. Carewicz, C. H. Mercier, A. Rode, N. Kinnersley, and P. Ward.** 2000. Efficacy and safety of oseltamivir in treatment of acute influenza: a randomised controlled trial. Lancet. **355:**1845-1850.

262. **Nicholson, K. J., A. E. Colegate, A. Podda, I. Stephenson, J. Wood, E. Ypma, and M. C. Zambon.** 2001. Confronting a potential H5N1 pandemic: a randomised controlled trial of conventional and MF59 adjuvanted influenza A/Duck/Singapore/97 (H5N3) surface antigen vaccine. Lancet:In Press.

263. **Novella, I. S., D. K. Clarke, J. Quer, E. A. Duarte, C. H. Lee, S. C. Weaver, S. F. Elena, A. Moya, E. Domingo, and J. J. Holland.** 1995. Extreme fitness differences in mammalian and insect hosts after continuous replication of vesicular stomatitis virus in sandfly cells. Journal of Virology. **69:**6805-6809.

264. **Okazaki, K., Y. Kawaoka, and R. G. Webster.** 1989. Evolutionary pathways of the PA genes of influenza A viruses. Virology. **172:**601-608.

265. **O'Neill, R. E., J. Talon, and P. Palese.** 1998. The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins. EMBO Journal. **17:**288-296.

266. **Oxford, J. S.** 2000. Influenza A pandemics of the 20th century with special reference to 1918: virology, pathology and epidemiology. Reviews in Medical Virology. **10:**119-133.

267. **Oxford, J. S.** 1975. Inhibition of the replication of influenza A and B viruses by a nucleoside analogue (ribovirin). Journal of General Virology. **28:**409-414.

268. **Oxford, J. S., I. S. Logan, and C. W. Potter.** 1970. *In vivo* selection of an influenza A2 strain resistant to amantadine. Nature. **226:**82-83.

269. **Pabico, R. C., R. G. Douglas, R. F. Betts, B. A. McKenna, and R. B. Freeman.** 1976. Antibody response to influenza vaccination in renal transplant patients: correlation with allograft function. Annals of Internal Medicine. **85:**431-436.

270. **Palache, A. M., R. Brands, and G. J. van Scharrenburg.** 1997. Immunogenicity and reactogenicity of influenza subunit vaccines produced in MDCK cells or fertilized chicken eggs. Journal of Infectious Diseases. **176:**S20-3.

271. **Palese, P., and R. W. Compans.** 1976. Inhibition of influenza virus replication in tissue culture by 2-deoxy-2,3-dehydro-*N*-trifluoroacetlyneuraminic acid (FANA): mechanism of action. Journal of General Virology. **33:**159-163.

272. **Park, E. K., M. R. Castrucci, A. Portner, and Y. Kawaoka.** 1998. The M2 ectodomain is important for its incorporation into influenza A virions. Journal of Virology. **72:**2449-2455.

273. **Park, K. Y., M. G. Lee, and J. C. Ryu.** 1997. Evolutionary stasis of M1 gene of human influenza A viruses and the possibility of their subtyping by restriction analysis of M1 gene polymerase chain reaction product. Acta Virologica. **41:**231-239.

274. **Patriarca, P. A., A. P. Kendal, P. C. Zakowski, N. J. Cox, M. S. Trautman, J. D. Cherry, D. M. Auerbach, J. McCusker, R. R. Belliveau, and K. D. Kappus.** 1984. Lack of significant person-to-person spread of swine influenza-like virus following fatal infection in an immunocompromised child. American Journal of Epidemiology. **119:**152-158.

275. **Payler, D. K., and P. A. Purdham.** 1984. Influenza A prophylaxis with amantadine in a boarding school. Lancet. **i:**502-504.

276. **Pekosz, A., and R. A. Lamb.** 1997. The CM2 protein of influenza C virus is an oligomeric integral membrane glycoprotein structurally analogous to influenza A virus M2 and influenza B virus NB proteins. Virology. **237:**439-451.

277. **Pemberton, R. M., R. Jennings, C. W. Potter, and J. S. Oxford.** 1986. Amantadine-resistance in clinical influenza A (H3N2) and (H1N1) virus isolates. Journal of Antimicrobial Chemotherapy. **18:**135-140.

278. **Penn, C. R.** 1998. Inhibitors of influenza virus neuraminidase, p. 477-487. *In* K. G. Nicholson and R. G. Webster and A. J. Hay (ed.), Textbook of Influenza. Blackwell Sciences, Oxford.

279. **Perales, B., and J. Ortin.** 1997. The influenza A virus PB2 polymerase subunit is required for the replication of viral RNA. Journal of Virology. **71:**1381-1385.

280. **Piller, S. C., G. D. Ewart, A. Premkumar, G. B. Cox, and P. W. Gage.** 1996. Vpr protein of human immunodeficiency virus type 1 forms cation-selective channels in planar lipid bilayers. Proceedings of the National Academy of Sciences USA. **93:**111-115.

281. **Pinto, L. H., G. R. Dieckmann, C. S. Gandhi, C. G. Papworth, J. Braman, M. A. Shaughnessy, J. D. Lear, R. A. Lamb, and W. F. DeGrado.** 1997. A functionally defined model for the M2 proton channel of

influenza A virus suggests a mechanism for its ion selectivity. Proceedings of the National Academy of Sciences USA. **94:**11301-11306.

282. **Pinto, L. H., L. J. Holsinger, and R. A. Lamb.** 1992. Influenza virus M2 protein has ion channel activity. Cell. **69:**517-528.

283. **Pinto, L. H., and R. A. Lamb.** 1995. Understanding the mechanism of action of the anti-influenza drug amantadine. Trends in Microbiology. **3:**271.

284. **Poch, O., I. Sauvaget, M. Delarue, and N. Tordo.** 1989. Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. EMBO Journal. **8:**3867-3874.

285. **Potter, C. W.** 1998. Chronicle of influenza pandemics, p. 3-18. *In* K. G. Nicholson and R. G. Webster and A. J. Hay (ed.), Textbook of Influenza. Blackwell Science, Oxford.

286. **Potter, C. W., and J. S. Oxford.** 1979. Determinants of immunity to influenza infection in man. British Medical Bulletin. **35:**69-75.

287. **Prud'homme, I. T., O. Zoueva, and J. M. Weber.** 1997. Amantadine susceptibility in influenza A virus isolates: determination methods and lack of resistance in a Canadian sample, 1991-94. Clinical and Diagnostic Virology. **8:**41-51.

288. **Qian, X. Y., F. Alonso-Caplen, and R. M. Krug.** 1994. Two functional domains of the influenza virus NS1 protein are required for regulation of nuclear export of mRNA. Journal of Virology. **68:**2433-2441.

289. **Qiu, Y., and R. M. Krug.** 1994. The influenza virus NS1 protein is a poly(A)-binding protein that inhibits nuclear export of mRNAs containing poly(A). Journal of Virology. **68:**2425-2432.

290. **Quer, J., R. Huerta, I. S. Novella, L. Tsimring, E. Domingo, and J. J. Holland.** 1996. Reproducible nonlinear population dynamics and critical points during replicative competitions of RNA virus quasispecies. Journal of Molecular Biology. **264:**465-471.

291. **Raad, I., J. Abbas, and E. Whimbey.** 1997. Infection control of nosocomial respiratory viral disease in the immunocompromised host. American Journal of Medicine. **102:**48-52.

292. **Rapicetta, M., C. Argentini, E. Spada, S. Dettori, M. P. Riccardi, and M. Toti.** 2000. Molecular evolution of HCV genotype 2c persistent infection following mother-to-infant transmission. Archives of Virology. **145:**965-977.

293. **Rasheed, S.** 1998. Infectivity and dynamics of HIV type 1 replication in the blood and reproductive tract of HIV type 1-infected women. AIDS Research and Human Retroviruses. **14:**105-118.

294. **Raymond, F. L., A. J. Caton, N. J. Cox, A. P. Kendal, and G. G. Brownlee.** 1986. The antigenicity and evolution of influenza H1 haemagglutinin, from 1950-1957 and 1977-1983: two pathways from one gene. Virology. **148:**275-287.

295. **Rayner, M. M., B. Cordova, and D. A. Jackson.** 1997. Population dynamics studies of wild-type and drug-resistant mutant HIV in mixed infections. Virology. **236:**85-94.

296. **Reay, P. A., I. M. Jones, F. M. Gotch, A. J. McMichael, and G. G. Brownlee.** 1989. Recognition of the PB1, neuraminidase, and matrix proteins of influenza virus A/NT/60/68 by cytotoxic T lymphocytes. Virology. **170:**477-485.

297. **Reid, A. H., T. G. Fanning, J. V. Hultin, and J. K. Taubenberger.** 1999. Origin and evolution of the 1918 "Spanish" influenza virus haemagglutinin gene. Proceedings of the National Academy of Sciences USA. **96:**1651-1656.

298. **Reid, A. H., T. G. Fanning, T. A. Janczewski, and J. K. Taubenberger.** 2000. Characterisation of the 1918 "Spanish" influenza virus neuraminidase gene. Proceedings of the National Academy of Sciences USA. **97:**6785-6790.

299. **Reid, A. H., J. K. Taubenberger, and T. G. Fanning.** 2001. The 1918 Spanish influenza: integrating history and biology. Microbes and Infection. **3:**81-87.

300. **Reuman, P. D., D. I. Bernstein, M. C. Keefer, E. C. Young, J. R. Sherwood, and G. M. Schlff.** 1989. Efficacy and safety of low dosage amantadine hydrochloride as prophylaxis for influenza A. Antiviral Research. **11:**27-40.

301. **Rima, B. K., J. A. P. Earle, K. Baczko, V. ter Meulen, U. G. Liebert, C. Carstens, J. Carabana, M. Caballero, M. L. Celma, and R. Fernandez-Munoz.** 1997. Sequence divergence of measles virus haemagglutinin during natural evolution and adaptation to cell culture. Journal of General Virology. **78:**97-106.

302. **Roberts, P. C., R. A. Lamb, and R. W. Compans.** 1998. The M1 and M2 proteins of influenza A virus are important determinants in filamentous particle formation. Virology. **240:**127-137.

303. **Robinson, H. L., L. A. Hunt, and R. G. Webster.** 1993. Protection against a lethal influenza virus challenge by immunisation with a haemagglutinin-expressing plasmid DNA. Vaccine. **11:**957-960.

304. **Rocha, E., N. J. Cox, R. A. Black, M. W. Harmon, C. J. Harrison, and A. P. Kendal.** 1991. Antigenic and genetic variation in influenza A (H1N1) virus isolates recovered from a persistently infected immunodeficient child. Journal of Virology. **65:**2340-2350.

305. **Rohm, C., N. Zhou, J. Suss, J. Mackenzie, and R. G. Webster.** 1996. Characterisation of a novel influenza haemagglutinin, H15: criteria for determination of influenza A subtypes. Virology. **217:**508-516.

306. **Rose, H. J.** 1980. The use of amantadine and influenza vaccine in a type A influenza epidemic in a boarding school. Journal of the Royal College of General Practitioners. **30:**619-621.

307. **Rose, H. J.** 1983. Use of amantadine in influenza: a second report. Journal of the Royal College of General Practitioners. **33:**651-653.

308. **Rowe, T., R. A. Abernathy, J. Hu-Primmer, W. W. Thompson, X. Lu, W. Lim, K. Fukuda, N. J. Cox, and J. M. Katz.** 1999. Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. Journal of Clinical Microbiology. **37:**937-943.

309. **Ruigrok, R. W.** 1998. Structure of influenza A, B and C viruses, p. 29-42. *In* K. G. Nicholson and R. G. Webster and A. J. Hay (ed.), Textbook of Influenza. Blackwell Sciences, Oxford.

310. **Ruigrok, R. W. H., E. M. A. Hirst, and A. J. Hay.** 1991. The specific inhibition of influenza A virus maturation by amantadine: an electron microscopic examination. Journal of General Virology. **72:**191-194.

311. **Sable, C. A., and F. G. Hayden.** 1995. Orthomyxoviral and paramyxoviral infections in transplant patients. Infectious Disease Clinics of North America. **9:**987-1003.

312. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science. **239:**487-491.

313. **Saito, T., Y. Kawaoka, and R. G. Webster.** 1993. Phylogenetic analysis of the N8 neuraminidase gene of influenza A viruses. Virology. **193:**868-876.

314. **Sambrook, J., E. F. Fritsch, and T. Manlatis.** 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbour Laboratory Press.

315. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences USA. **74:**5463-5467.

316. **Sansom, M. S., I. D. Kerr, G. R. Smith, and H. S. Son.** 1997. The influenza A virus M2 channel: a molecular modelling and simulation study. Virology. **233:**163-173.

317. **Scholtissek, C.** 1994. Source for influenza pandemics. European Journal of Epidemiology. **10:**455-458.

318. **Schoitissek, C., H. Burger, O. Kistner, and K. F. Shortridge.** 1985. The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. Virology. **147:**287-94.

319. **Scholtissek, C., and R. G. Webster.** 1998. Long-term stability of the anti-influenza A compounds--amantadine and rimantadine. Antiviral Research. **38:**213-5.

320. **Schroeder, C., C. M. Ford, S. A. Wharton, and A. J. Hay.** 1994. Functional reconstitution in lipid vesicles of influenza virus M2 protein expressed by baculovirus: evidence for proton transfer activity. Journal of General Virology. **75:**3477-3484.

321. **Schweighofer, K. J., and A. Pohorille.** 2000. Computer simulation of ion channel gating: the M(2) channel of influenza A virus in a lipid bilayer. Biophysical Journal. **78:**150-163.

322. **Shapiro, G. I., T. Gurney, Jr., and R. M. Krug.** 1987. Influenza virus gene expression: control mechanisms at early and late times of infection and nuclear-cytoplasmic transport of virus-specific RNAs. Journal of Virology. **61:**764-773.

323. **Shaw, M. W., P. W. Choppin, and R. A. Lamb.** 1983. A previously unrecognised influenza B virus glycoprotein from a bicistronic mRNA that also encodes the viral neuraminidase. Proceedings of the National Academy of Sciences USA. **80:**4879-4883.

324. **Shimbo, K., D. L. Brassard, R. A. Lamb, and L. H. Pinto.** 1996. Ion selectivity and activation of the M2 ion channel of influenza virus. Biophysical Journal. **70:**1335-1346.

325. **Shope, R.** 1934. The infection of ferrets with swine influenza virus. Journal of Experimental Medicine. **62:**49-61.

326. **Shope, R. E.** 1931. Swine influenza. I. Experimental transmission and pathology. Journal of Experimental Medicine. **54:**373-385.

327. **Shope, R. E.** 1931. Swine influenza. III. Filtration experiments and etiology. Journal of Experimental Medicine. **54:**373-385.

328. **Shu, L. L., W. J. Bean, and R. G. Webster.** 1993. Analysis of the evolution and variation of the human influenza A virus nucleoprotein gene from 1933 to 1990. Journal of Virology. **67:**2723-2729.

329. **Shuck, K., R. A. Lamb, and L. H. Pinto.** 2000. Analysis of the pore structure of the influenza A virus M2 ion channel by the substituted-cysteine accessibility method. Journal of Virology. **74:**7755-7761.

330. **Sidweii, R. W., D. F. Smee, J. H. Huffman, D. L. Barnard, K. W. Bailey, J. D. Morrey, and Y. S. Babu.** 2001. *In vivo* influenza virus-inhibitory effects of the cyclopentane neuraminidase inhibitor RWJ-270201. Antimicrobial Agents and Chemotherapy. **45:**749-757.

331. **Slepushkin, V. A., J. M. Katz, R. A. Black, W. C. Gamble, P. A. Rota, and N. J. Cox.** 1995. Protection of mice against influenza A virus challenge by vaccination with baculovirus-expressed M2 protein. Vaccine. **13:**1399-1402.

332. **Smee, D. F., J. H. Huffman, A. C. Morrison, D. L. Barnard, and R. W. Sidwell.** 2001. Cyclopentane neuraminidase inhibitors with potent *in vitro* anti-influenza virus activities. Antimicrobial Agents and Chemotherapy. **45:**743-748.

333. **Smith, D. B., J. McAlister, C. Casino, and P. Simmonds.** 1997. Virus 'quasispecies': making a mountain out of a molehill. Journal of General Virology. **78:**1511-1519.

334. **Smith, W.** 1935. Cultivation of the virus of influenza. British Journal of Experimental Pathology. **16:**508-512.

335. **Smith, W., C. H. Andrewes, and P. P. Laidlaw.** 1933. A virus obtained from influenza patients. Lancet. **ii:**66-68.

336. **Smith, W., C. H. Andrewes, and C. H. Stuart-Harris.** 1938. The immunisation of human volunteers. Medical Research Council Special Report Series. **228:**137-144.

337. **Sommer, G., S. Günther, M. Sterneck, S. Otto, and H. Will.** 1997. A new class of defective hepatitis B genomes with an internal poly(dA) sequence. Virology. **239:**402-412.

338. **Steinhauer, D. A., E. Domingo, and J. J. Holland.** 1992. Lack of evidence for proofreading mechanisms associated with an RNA virus polymerase. Gene. **122:**281-288.

339. **Steinhauer, D. A., and S. A. Wharton.** 1998. Structure and function of the haemagglutinin, p. 54-64. *In* K. J. Nicholson and R. G. Webster and A. J. Hay (ed.), Textbook of Influenza. Blackwell Science, Oxford.

340. **Steinhauer, D. A., S. A. Wharton, J. J. Skehel, D. C. Wiley, and A. J. Hay.** 1991. Amantadine selection of a mutant influenza virus containing an acid-stable haemagglutinin glycoprotein: evidence for virus-specific regulation of the pH of glycoprotein transport vesicles. Proceedings of the National Academy of Sciences USA. **88:**11525-11529.

341. **Stieneke-Grober, A., M. Vey, H. Angliker, E. Shaw, G. Thomas, C. Roberts, H. D. Klenk, and W. Garten.** 1992. Influenza virus haemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. EMBO Journal. **11:**2407-2414.

342. **Stockton, J. D., and M. C. Zambon.** 1999. The influenza virus. Hospital Medicine. **60:**724-730.

343. **Stuart-Harris, C. H., G. C. Schild, and J. S. Oxford.** 1985. The structure and replication of influenza viruses. *In* C. H. Stuart-Harris and G. C. Schild and J. S. Oxford (ed.), Influenza: the viruses and the disease, 2nd ed. London, Arnold.

344. **Sugaya, N., K. Nerome, M. ishida, R. Nerome, M. Nagae, Y. Takeuchi, and M. Osano.** 1992. Impact of influenza virus infections as a cause of pediatric hospitalisation. Journal of Infectious Diseases. **165:**373-375.

345. **Sugita, S., Y. Yoshioka, S. Itamura, Y. Kanegae, K. Oguchi, T. Gojobori, K. Nerome, and A. Oya.** 1991. Molecular evolution of haemagglutinin genes of H1N1 swine and human influenza A viruses. Journal of Molecular Evolution. **32:**16-23.

346. **Sugrue, R. J., G. Bahadur, M. C. Zambon, M. Hail-Smith, A. R. Douglas, and A. J. Hay.** 1990. Specific structural alteration of the influenza haemagglutinin by amantadine. EMBO Journal. **9:**3469-3476.

347. **Sugrue, R. J., R. B. Belshe, and A. J. Hay.** 1990. Palmitoylation of the influenza virus M2 protein. Virology. **179:**51-56.

348. **Sugrue, R. J., and A. J. Hay.** 1991. Structural characteristics of the M2 protein of influenza A viruses: evidence that it forms a tetrameric channel. Virology. **180:**617-624.

349. **Sunstrom, N. A., L. S. Premkumar, A. Premkumar, G. Ewart, G. B. Cox, and P. W. Gage.** 1996. Ion channels formed by NB, an influenza B virus protein. Journal of Membrane Biology. **150:**127-132.

350. **Sweet, C., F. G. Hayden, K. J. Jakeman, S. Grambas, and A. J. Hay.** 1991. Virulence of rimantadine-resistant human influenza A (H3N2) viruses in ferrets. Journal of Infectious Diseases. **164:**969-972.

351. **Tablan, O. C., L. J. Anderson, N. H. Arden, R. F. Breiman, J. C. Butler, and M. M. McNeil.** 1994. Guideline for prevention of nosocomial pneumonia. Part 1. Issues on prevention of nosocomial pneumonia-1994. American Journal of Infection Control. **22:**247-292.

352. **Tai, C. Y., P. A. Escarpe, R. W. Sidweli, M. A. Williams, W. Lew, H. Wu, C. U. Kim, and D. B. Mendel.** 1998. Characterisation of human influenza virus variants selected *in vitro* in the presence of the neuraminidase inhibitor GS 4071. Antimicrobial Agents and Chemotherapy. **42:**3234-3241.

353. **Talon, J., M. Salvatore, R. E. O'Neill, Y. Nakaya, H. Zheng, T. Muster, A. Garcia-Sastre, and P. Palese.** 2000. Influenza A and B viruses expressing altered NS1 proteins: a vaccine approach. Proceedings of the National Academy of Sciences U S A. **97:**4309-4314.

354. **Taubenberger, J. K., A. H. Reid, A. E. Krafft, K. E. Bijwaard, and T. G. Fanning.** 1997. Initial genetic characterisation of the 1918 "Spanish" influenza virus. Science. **275:**1793-1796.

355. **Tebas, P., D. Scholl, J. Joliick, K. McHarg, M. Arens, and P. D. Olivo.** 1998. A rapid assay to screen for drug-resistant herpes simplex virus. Journal of Infectious Diseases. **177:**217-220.

356. **Tenover, F. C., M. V. Lancaster, B. C. Hill, C. D. Steward, S. A. Stocker, G. A. Hancock, C. M. O'Hara, S. K. McAllister, N. C. Clark, and K. Hiramatsu.** 1998. Characterisation of staphylococci with reduced susceptibilities to vancomycin and other glycopeptides. Journal of Clinical Microbiology. **36:**1020-1027.

357. **Terwilliger, E. F., E. A. Cohen, Y. C. Lu, J. G. Sodroski, and W. A. Haseltine.** 1989. Functional role of human immunodeficiency virus type 1 vpu. Proceedings of the National Academy of Sciences USA. **86:**5163-5167.

358. **Thomas, J. M., M. P. Stevens, N. Percy, and W. S. Barclay.** 1998. Phosphorylation of the M2 protein of influenza A virus is not essential for virus viability. Virology. **252:**54-64.

359. **Tobler, K., M. L. Kelly, L. H. Pinto, and R. A. Lamb.** 1999. Effect of cytoplasmic tail truncations on the activity of the M2 ion channel of influenza A virus. Journal of Virology. **73:**9695-9701.

360. **Tosteson, M. T., L. H. Pinto, L. J. Holsinger, and R. A. Lamb.** 1994. Reconstitution of the influenza virus M2 ion channel in lipid bilayers. Journal of Membrane Biology. **142:**117-126.

361. **Treanor, J. J., F. G. Hayden, P. S. Vrooman, R. Barbarash, R. Bettis, D. Riff, S. Singh, N. Kinnersley, P. Ward, and R. G. Mills.** 2000. Efficacy and safety of the oral neuraminidase inhibitor oseltamivir in treating acute influenza: a randomised controlled trial. Journal of the American Medical Association. **283:**1016-1024.

362. **Treanor, J. J., E. L. Tierney, S. L. Zebedee, R. A. Lamb, and B. R. Murphy.** 1990. Passively transferred monoclonal antibody to the M2 protein inhibits influenza A virus replication in mice. Journal of Virology. **64:**1375-1377.

363. **Ulmer, J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felgner, V. J. Dwarki, S. H. Gromkowski, R. R. Deck, C. M. DeWitt, A. Friedman, L. A. Hawe, K. R. Leander, D. Martinez, H. C. Perry, J. W. Shiver, D. L. Montgomery, and M. A. Liu.** 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science. **259:**1745-1749.

364. **Valette, M., J. P. Allard, M. Aymard, and V. Millet.** 1993. Susceptibilities to rimantadine of influenza A/H1N1 and A/H3N2 viruses isolated during the epidemics of 1988 to 1989 and 1989 to 1990. Antimicrobial Agents and Chemotherapy. **37:**2239-2240.

365. **van Hartesveidt, F. R.** 1992. , p. 1-12. *In* F. R. van Hartesveldt (ed.), The 1918-1919 pandemic of influenza: the urban impact in the western world. Edwin Millen Press, New York.

366. **Vaniandschoot, P., E. Beirnaert, J. Grooten, W. M. Jou, and W. Fiers.** 1998. pH-dependent aggregation and secretion of soluble monomeric influenza haemagglutinin. Archives of Virology. **143:**227-239.

367. **van't Wout, A. B., L. J. Ran, C. L. Kuiken, N. A. Kootstra, S. T. Pals, and H. Schuitemaker.** 1998. Analysis of the temporal relationship between

human immunodeficiency virus type 1 quasispecies in sequential blood samples and various organs obtained at autopsy. Journal of Virology. **72:**488-496.

368. **Varghese, J. N., P. M. Colman, A. van Donkelaar, T. J. Blick, A. Sahasrabudhe, and J. L. McKimm-Breschkin.** 1997. Structural evidence for a second sialic acid binding site in avian influenza virus neuraminidases. Proceedings of the National Academy of Sciences USA. **94:**11808-11812.
369. **Varghese, J. N., W. G. Laver, and P. M. Colman.** 1983. Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9 Å resolution. Nature. **303:**35-40.
370. **Veenstra, J., R. Schuurman, M. Cornellssen, A. B. van't Wout, C. A. Boucher, H. Schuitemaker, J. Goudsmit, and R. A. Coutinho.** 1995. Transmission of zidovudine-resistant human immunodeficiency virus type 1 variants following deliberate injection of blood from a patient with AIDS: characteristics and natural history of the virus. Clinical Infectious Diseases. **21:**556-560.
371. **Veit, M., H. D. Klenk, A. Kendal, and R. Rott.** 1991. The M2 protein of influenza A virus is acylated. Journal of General Virology. **72:**1461-1465.
372. **Virelizier, J. L.** 1975. Host defences against influenza virus: the role of anti-haemagglutinin antibody. Journal of Immunology. **115:**434-439.
373. **Voeten, J. T. M., R. Brands, A. M. Palache, G. J. M. van Scharrenburg, G. F. Rimmelzwaan, A. D. M. E. Osterhaus, and E. C. J. Claas.** 1999. Characterisation of high-growth reassortant influenza A viruses generated in MDCK cells cultured in serum-free medium. Vaccine. **17:**1942-1950.
374. **von Itzstein, M., W. Y. Wu, G. B. Kok, M. S. Pegg, J. C. Dyason, B. Jin, T. Van Phan, M. L. Smythe, H. F. White, S. W. Oliver, and et ai.** 1993. Rational design of potent sialidase-based inhibitors of influenza virus replication. Nature. **363:**418-423.
375. **Wang, C., R. A. Lamb, and L. H. Pinto.** 1995. Activation of the M2 ion channel of influenza virus: a role for the transmembrane domain histidine residue. Biophysical Journal. **69:**1363-1371.
376. **Wang, C., R. A. Lamb, and L. H. Pinto.** 1994. Direct measurement of the influenza A virus M2 protein ion channel activity in mammalian cells. Virology. **205:**133-140.

377. **Wang, W., W. M. Lee, A. G. Mosser, and R. R. Rueckert.** 1998. WIN 52035-dependent human rhinovirus 16: assembly deficiency caused by mutations near the canyon surface. Journal of Virology. **72:**1210-1218.

378. **Ward, A. C., L. A. Castelli, A. C. Lucantoni, J. F. White, A. A. Azad, and I. G. Macreadie.** 1995. Expression and analysis of the NS2 protein of influenza A virus. Archives of Virology. **140:**2067-2073.

379. **Watanabe, K., H. Handa, K. Mizumoto, and K. Nagata.** 1996. Mechanism for inhibition of influenza virus RNA polymerase activity by matrix protein. Journal of Virology. **70:**241-247.

380. **Webster, R. G.** 1998. DNA vaccination: a potential future strategy, p. 410-421. *In* K. G. Nicholson and R. G. Webster and A. J. Hay (ed.), Textbook of Influenza. Blackwell Sciences, Oxford.

381. **Webster, R. G.** 1999. Potential advantages of DNA immunisation for influenza epidemic and pandemic planning. Clinical Infectious Diseases. **28:**225-229.

382. **Webster, R. G., and W. J. Bean Jr.** 1998. Evolution and ecology of influenza viruses: interspecies transmission, p. 109-125. *In* K. G. Nicholson and R. G. Webster and A. J. Hay (ed.), The Textbook of Influenza. Blackwell, Oxford.

383. **Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka.** 1992. Evolution and ecology of influenza A viruses. Microbiological Reviews. **56:**152-179.

384. **Webster, R. G., Y. Kawaoka, W. J. Bean, C. W. Beard, and M. Brugh.** 1985. Chemotherapy and vaccination: a possible strategy for the control of highly virulent influenza virus. Journal of Virology. **55:**173-176.

385. **Wendt, C. H., D. J. Weisdorf, M. C. Jordan, H. H. Balfour, and M. I. Hertz.** 1992. Parainfluenza virus respiratory infection after bone marrow transplantation. New England Journal of Medicine. **326:**921-926.

386. **Wentworth, B. B., and L. French.** 1970. Plaque assay of cytomegalovirus strains of human origin. Proceedings of the Society of Experimental Biology and Medicine. **135:**253-258.

387. **Whimbey, E., and G. P. Bodey.** 1992. Viral pneumonia in the immunocompromised adult with neoplastic disease: the role of common community respiratory viruses. Seminars in Respiratory Infections. **7:**122-131.

388. **Whimbey, E., R. E. Champlin, R. B. Couch, J. A. Englund, J. M. Goodrich, I. Raad, D. Przepiorka, V. A. Lewis, N. Mirza, H. Yousuf, J. J. Tarrand, and G. P. Bodey.** 1996. Community respiratory virus infections among hospitalised adult bone marrow transplant recipients. Clinical Infectious Diseases. **22:**778-782.

389. **Whiting, P., C. A. Joseph, M. Zambon, M. Nunn, D. Fleming, and J. M. Watson.** 1999. Influenza activity and surveillance in England and Wales: October 1998 to June 1999. Communicable Disease and Public Health. **2:**273-279.

390. **Whitley, R. J.** 1997. Herpes simplex virus, p. 375-410. *In* D. D. Richman and R. J. Whitley and F. G. Hayden (ed.), Clinical Virology. Churchill Livingston Inc., NY.

391. **WHO.** 1996. WHO influenza surveillance. Weekly Epidemiological Record. **71:**353-357.

392. **Wiley, D. C., and J. J. Skehel.** 1987. The structure and function of the haemagglutinin membrane glycoprotein of influenza virus. Annual Review of Biochemistry. **56:**365-394.

393. **Wiley, D. C., I. A. Wilson, and J. J. Skehel.** 1981. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. Nature. **289:**373-378.

394. **Williams, M. A., and R. A. Lamb.** 1986. Determination of the orientation of an integral membrane protein and sites of glycosylation by oligonucleotide-directed mutagenesis: influenza B virus NB glycoprotein lacks a cleavable signal sequence and has an extracellular NH2-terminal region. Molecular and Cellular Biology. **6:**4317-4328.

395. **Williamson, J. C., and P. S. Pegram.** 2000. Respiratory distress associated with zanamivir. New England Journal of Medicine. **342:**661-662.

396. **Wilson, I. A., J. J. Skehel, and D. C. Wiley.** 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. Nature. **289:**366-373.

397. **Winter, G., and S. Fields.** 1980. Cloning of influenza cDNA ino M13: the sequence of the RNA segment encoding the A/PR/8/34 matrix protein. Nucleic Acids Research. **8:**1965-1974.

398. **Wood, J. M., R. C. Bethell, J. A. Coates, N. Healy, S. A. Hiscox, B. A. Pearson, D. M. Ryan, J. Ticehurst, J. Tilling, S. M. Walcott, and et al.** 1993. 4-Guanidino-2,4-dideoxy-2,3-dehydro-N-acetylneuraminic acid is a

highly effective inhibitor both of the sialidase (neuraminidase) and of growth of a wide range of influenza A and B viruses *in vitro*. Antimicrobial Agents and Chemotherapy. **37:**1473-1479.

399. **Wood, J. M., and M. S. Williams.** 1998. History of inactivated influenza vaccines, p. 317-323. *In* K. G. Nicholson and R. G. Webster and A. J. Hay (ed.), Textbook of Influenza. Blackwell Sciences, Oxford.
400. **Wrigley, N. G., J. J. Skehel, P. A. Charlwood, and C. M. Brand.** 1973. The size and shape of influenza virus neuraminidase. Virology. **51:**525-529.
401. **Xu, X., N. J. Cox, C. A. Bender, H. L. Regnery, and M. W. Shaw.** 1996. Genetic variation in neuraminidase genes of influenza A (H3N2) viruses. Virology. **224:**175-183.
402. **Yasuda, J., D. J. Bucher, and A. Ishihama.** 1994. Growth control of influenza A virus by M1 protein: analysis of transfectant viruses carrying the chimeric M gene. Journal of Virology. **68:**8141-8146.
403. **Yasuda, J., S. Nakada, A. Kato, T. Toyoda, and A. Ishihama.** 1993. Molecular assembly of influenza virus: association of the NS2 protein with virion matrix. Virology. **196:**249-255.
404. **Yasuda, J., T. Toyoda, M. Nakayama, and A. Ishihama.** 1993. Regulatory effects of matrix protein variations on influenza virus growth. Archives of Virology. **133:**283-294.
405. **Yerly, S., L. Kaiser, E. Race, J. P. Bru, F. Clavei, and L. Perrin.** 1999. Transmission of antiretroviral-drug-resistant HIV-1 variants. Lancet. **354:**729-733.
406. **Yoshimura, A., K. Kuroda, K. K. Kawasaki, S. Yamashina, T. Maeda, and S. i. Ohnishi.** 1982. Infectious cell entry mechanism of influenza virus. Journal of Virology. **43:**284-293.
407. **Young, D.** 2001. RWJ-270201: a novel neuraminidase inhibitor for influenza. The Origin and Control of Pandemic Influenza. The Royal Society, London.
408. **Yousuf, H. M., J. Englund, R. Couch, K. Rolston, M. Luna, J. Goodrich, V. Lewis, N. Q. Mirza, M. Andreeff, C. Koller, L. Elting, G. P. Bodey, and E. Whimbey.** 1997. Influenza among hospitalised adults with leukaemia. Clinical Infectious Diseases. **24:**1095-1099.
409. **Zambon, M.** 1998. Laboratory diagnosis of influenza, p. 291-313. *In* K. G. Nicholson and R. G. Webster and A. J. Hay (ed.), Textbook of Influenza. Blackwell Sciences, Oxford.

410. **Zebedee, S. L., and R. A. Lamb.** 1989. Growth restriction of influenza A virus by M2 protein antibody is genetically linked to the M1 protein. Proceedings of the National Academy of Sciences USA. **86:**1061-1065.

411. **Zebedee, S. L., and R. A. Lamb.** 1988. Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. Journal of Virology. **62:**2762-2772.

412. **Zebedee, S. L., and R. A. Lamb.** 1989. Nucleotide sequences of influenza A virus RNA segment 7: a comparison of five isolates. Nucleic Acids Research. **17:**2870.

413. **Zhirnov, O. P.** 1992. Isolation of matrix protein M1 from influenza viruses by acid-dependent extraction with nonionic detergent. Virology. **186:**324-330.

414. **Zhirnov, O. P.** 1990. Solubilisation of matrix protein M1/M from virions occurs at different pH for orthomyxo- and paramyxoviruses. Virology. **176:**274-279.

415. **Zhirnov, O. P., and H. D. Klenk.** 1997. Histones as a target for influenza virus matrix protein M1. Virology. **235:**302-310.

416. **Zhong, Q., D. M. Newns, P. Pattnaik, J. D. Lear, and M. L. Klein.** 2000. Two possible conducting states of the influenza A virus M2 ion channel. FEBS Letters. **473:**195-198.

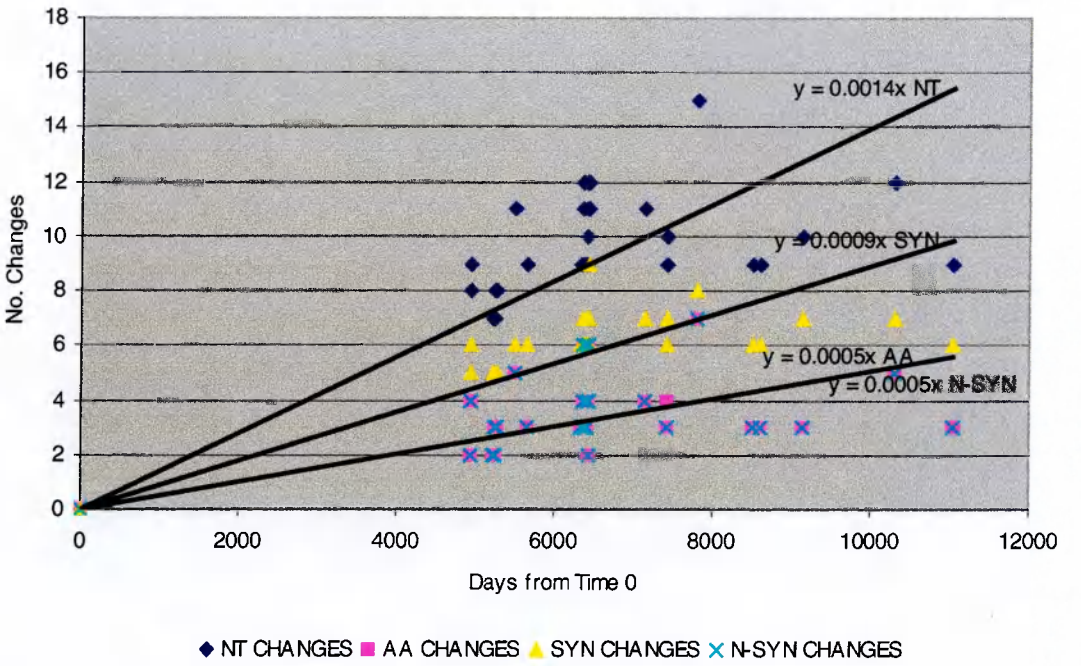
417. **Ziegler, T., M. L. Hemphili, M. L. Ziegler, G. Perez-Oronoz, A. I. Klimov, A. W. Hampson, H. L. Regnery, and N. J. Cox.** 1999. Low incidence of rimantadine-resistance in field isolates of influenza A viruses. Journal of Infectious Diseases. **180:**935-939.

418. **Zlydnikov, D. M., O. I. Kubar, T. P. Kovaleva, and L. E. Kamforin.** 1981. Study of rimantadine in the USSR: a review of the literature. Reviews of Infectious Diseases. **3:**408-421.

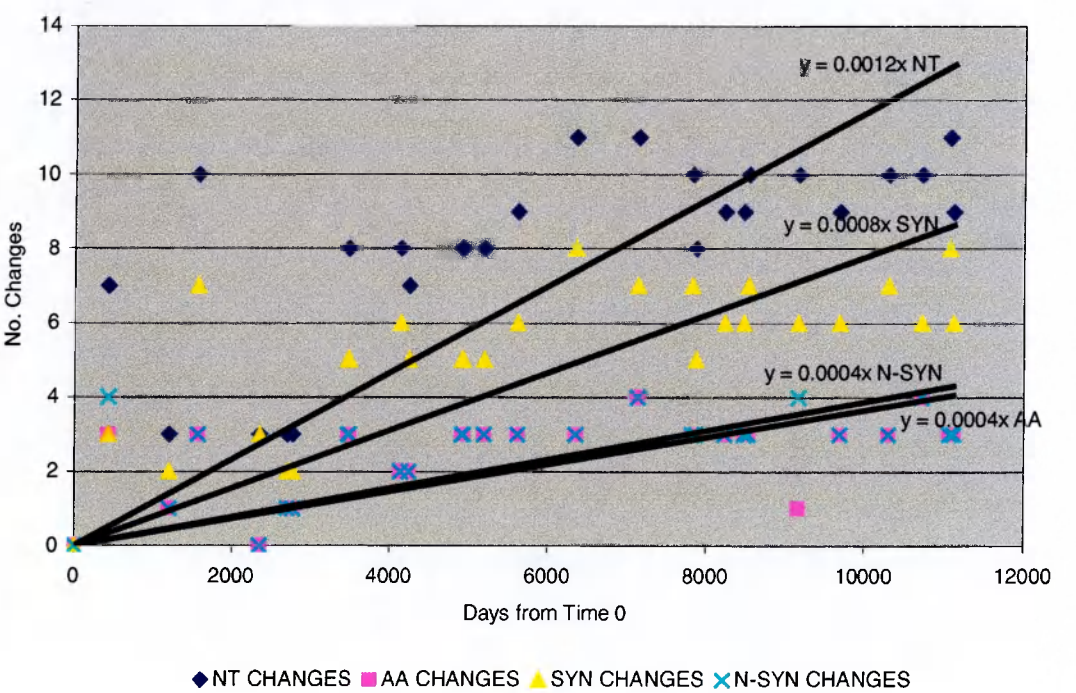
419. **Zweerink, H. J., S. A. Courtneidge, J. J. Skehel, M. J. Crumpton, and B. A. Askonas.** 1977. Cytotoxic T cells kill influenza virus infected cells but do not distinguish between serologically distinct type A viruses. Nature. **267:**354-356.

Appendix

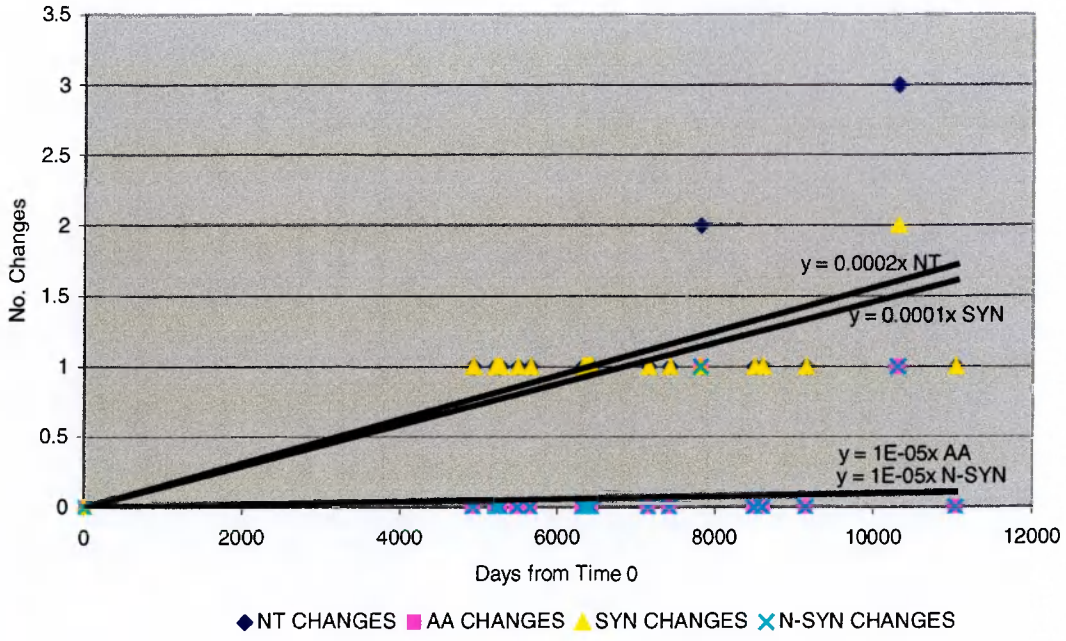
APPENDIX 1 Rate of change of M2 of resistant viruses (H3N2)



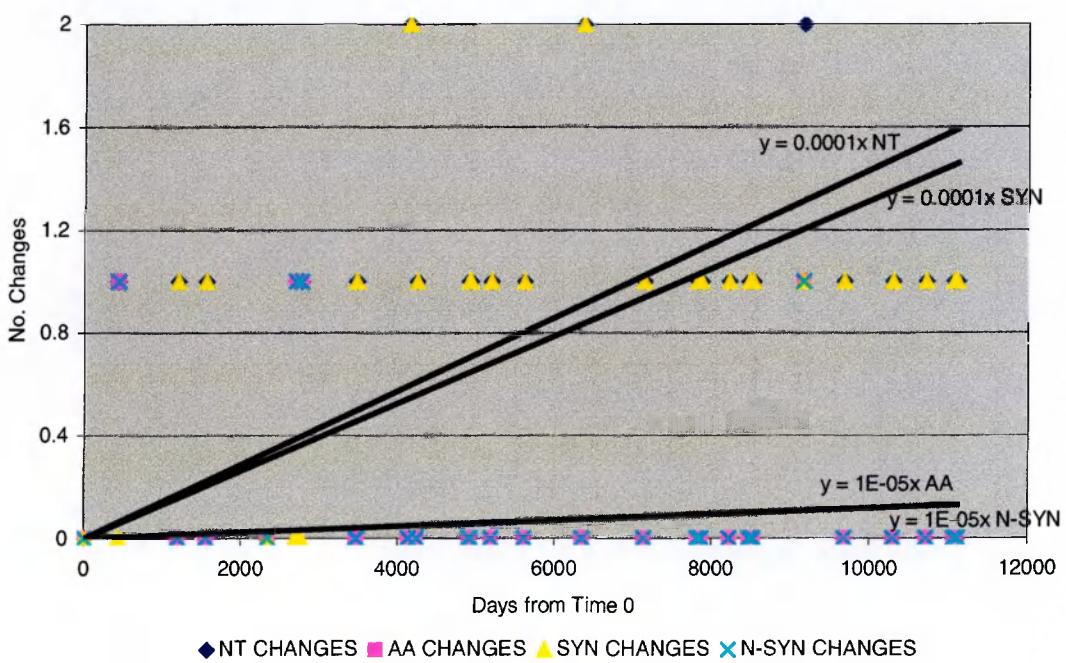
APPENDIX 2 Rate of change of M2 from sensitive viruses (H3N2)



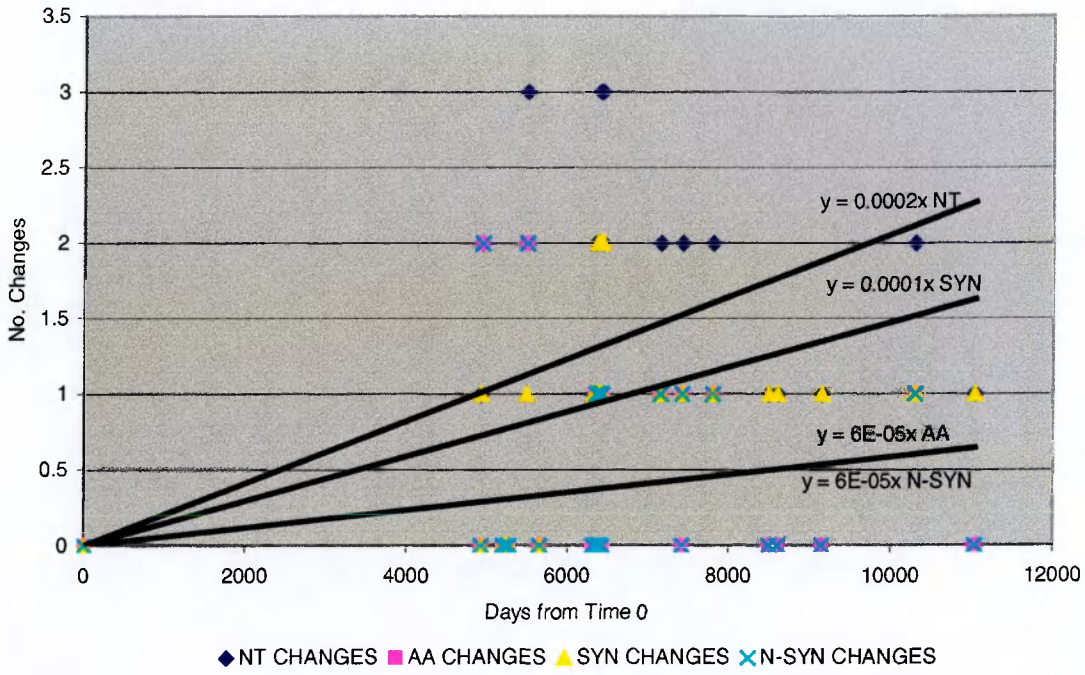
APPENDIX 3 Rate of change of M2 extracellular domain from resistant viruses (H3N2)



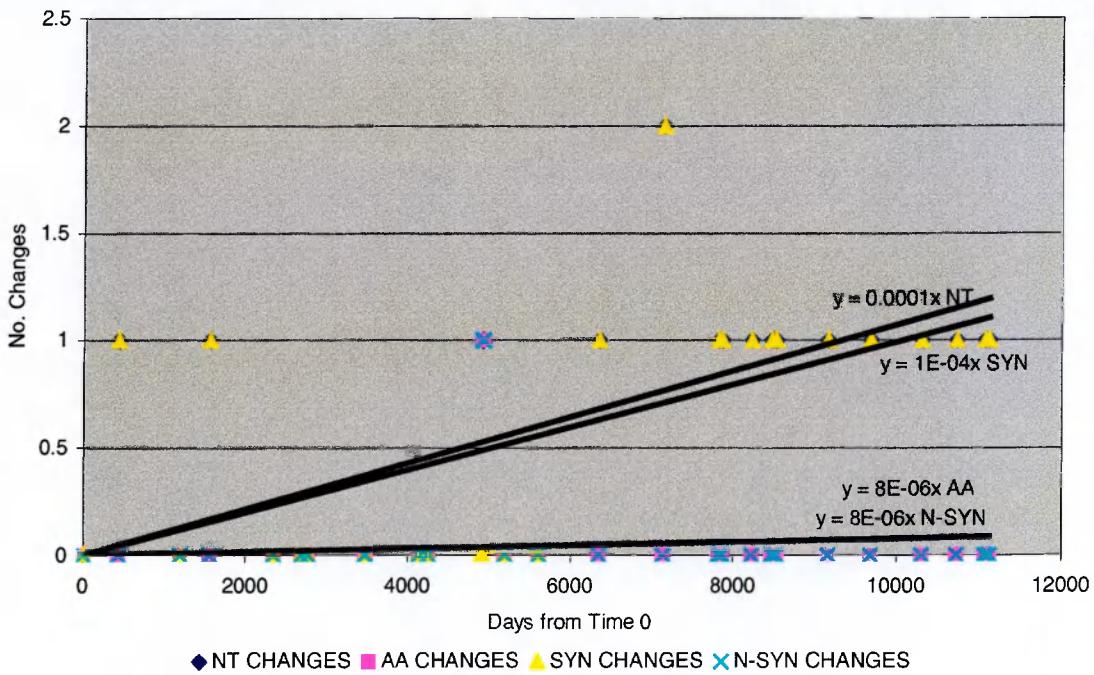
APPENDIX 4 Rate of change of M2 extracellular domain from sensitive viruses (H3N2)



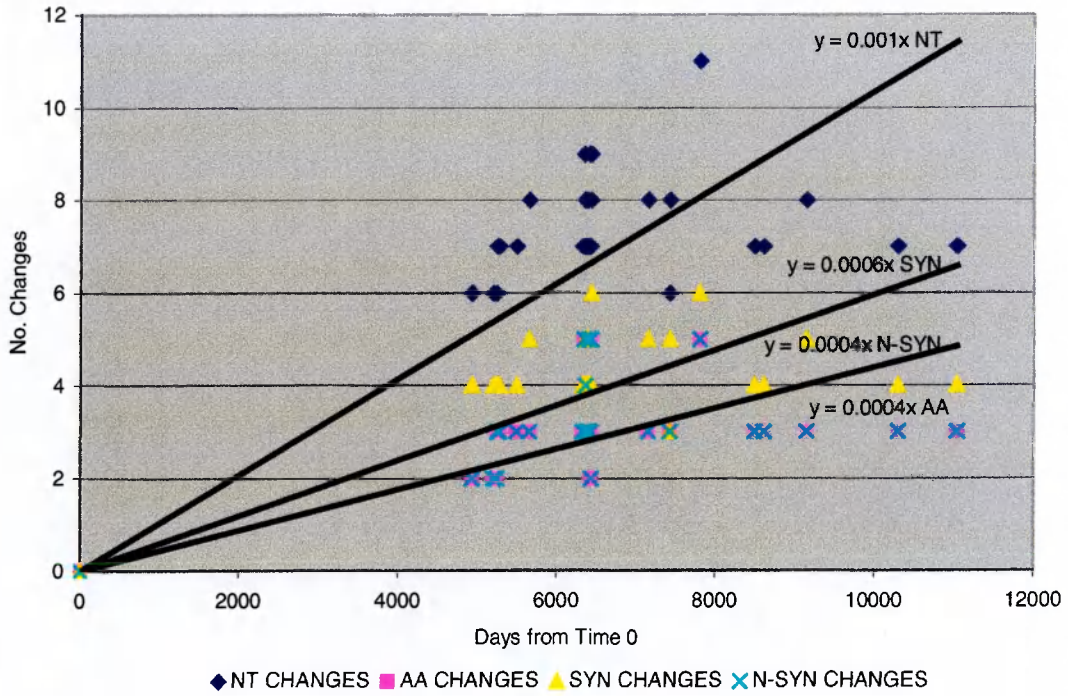
APPENDIX 5 Rate of change of M2 transmembrane domain from resistant viruses (H3N2)



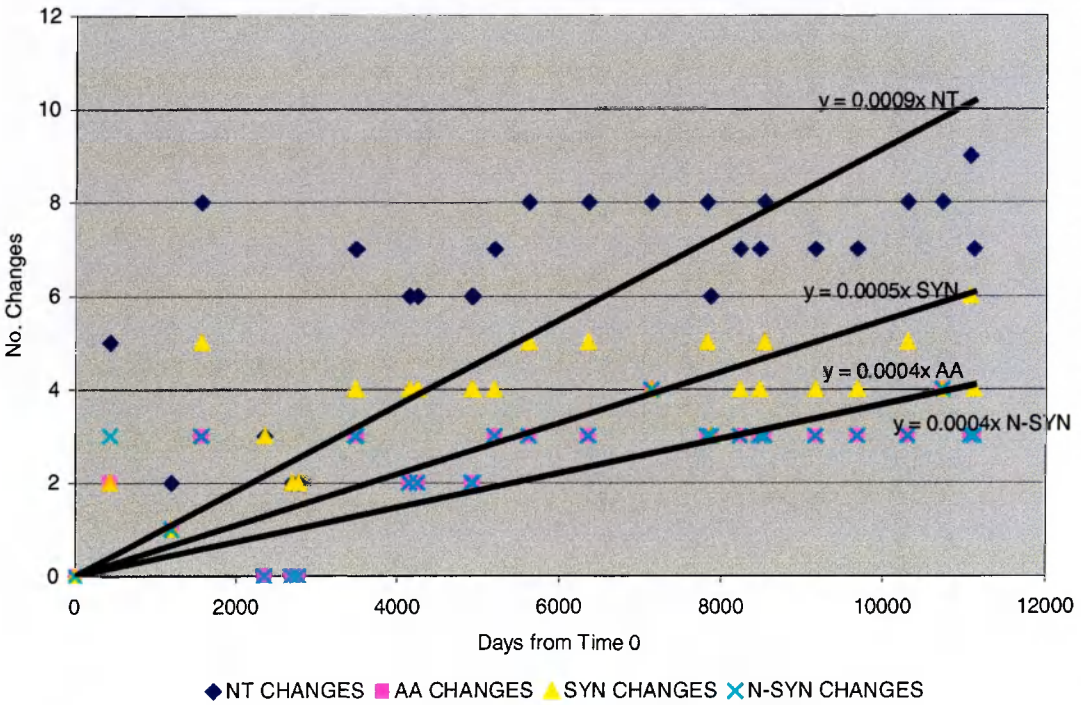
APPENDIX 6 Rate of change of M2 transmembrane domain from sensitive viruses (H3N2)



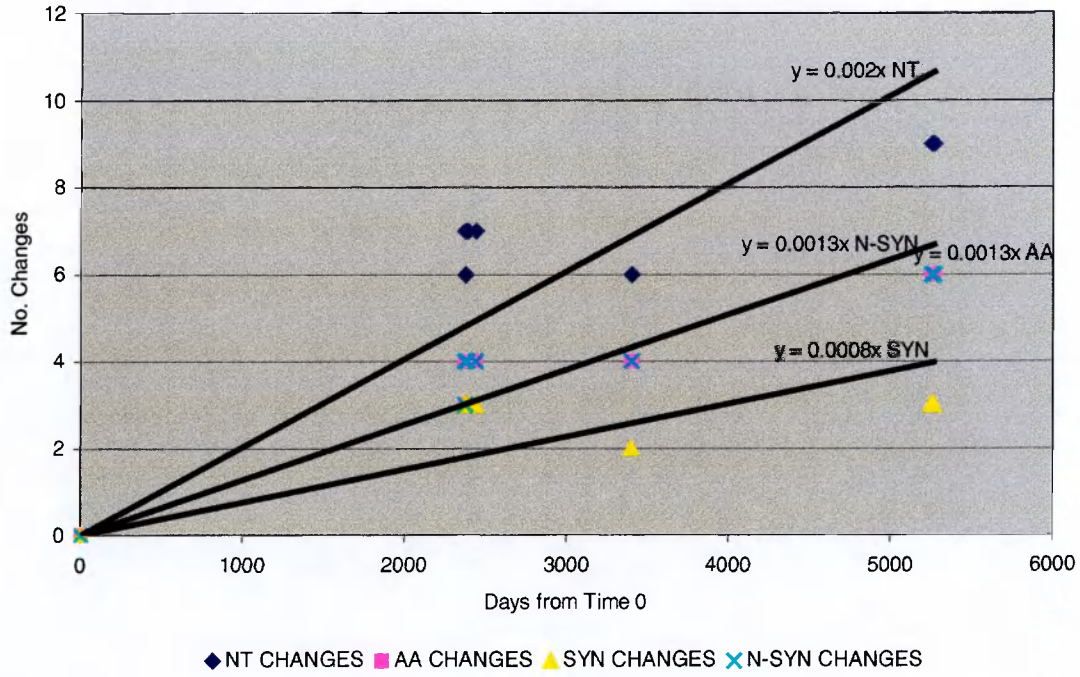
APPENDIX 7 Rate of change of M2 cytoplasmic domain from resistant viruses (H3N2)



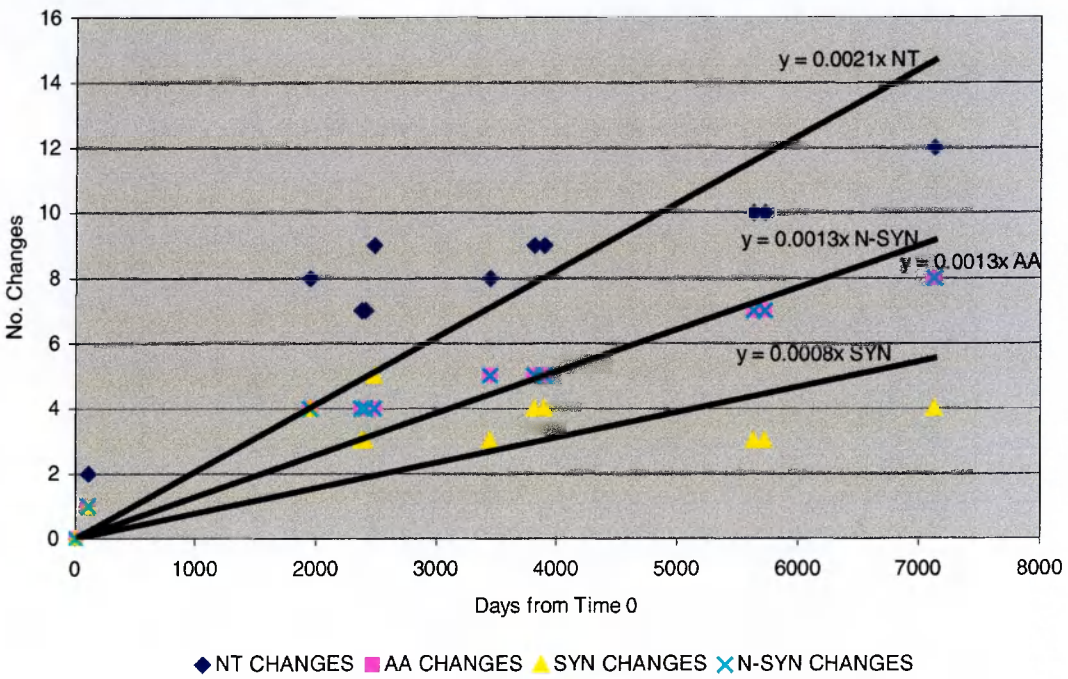
APPENDIX 8 Rate of change of M2 cytoplasmic domain from sensitive viruses (H3N2)



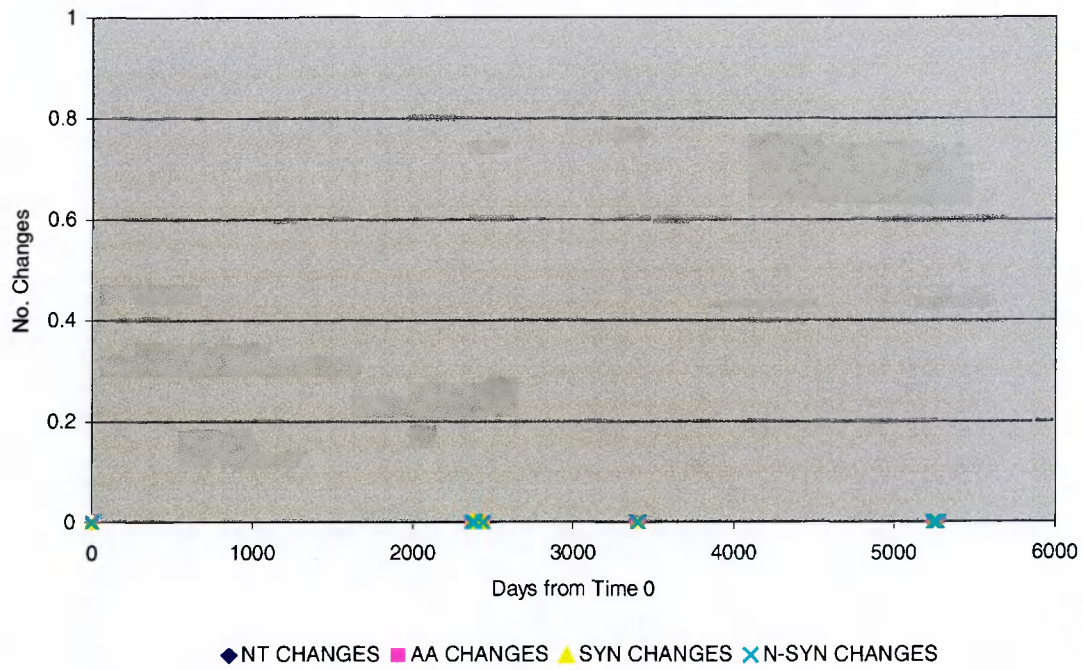
APPENDIX 9 Rate of change of M2 from resistant viruses (H1N1)



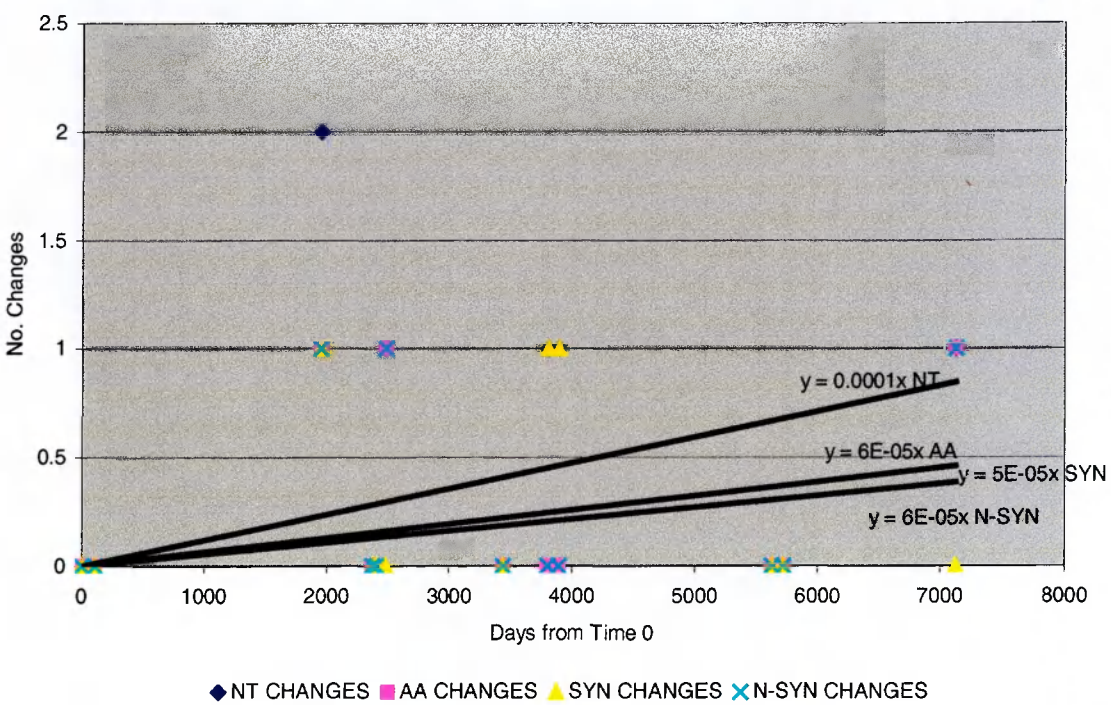
APPENDIX 10 Rate of change of M2 from sensitive viruses (H1N1)



APPENDIX 11 Rate of change of M2 extracellular domain from resistant viruses (H1N1)



APPENDIX 12 Rate of change of M2 extracellular domain from sensitive viruses (H1N1)



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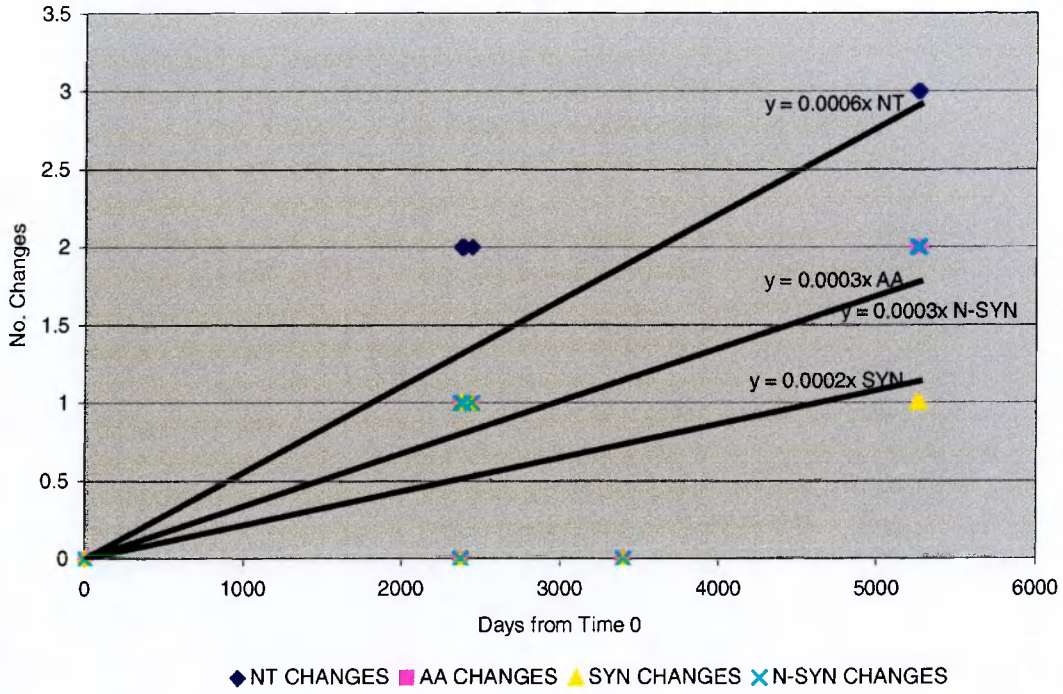
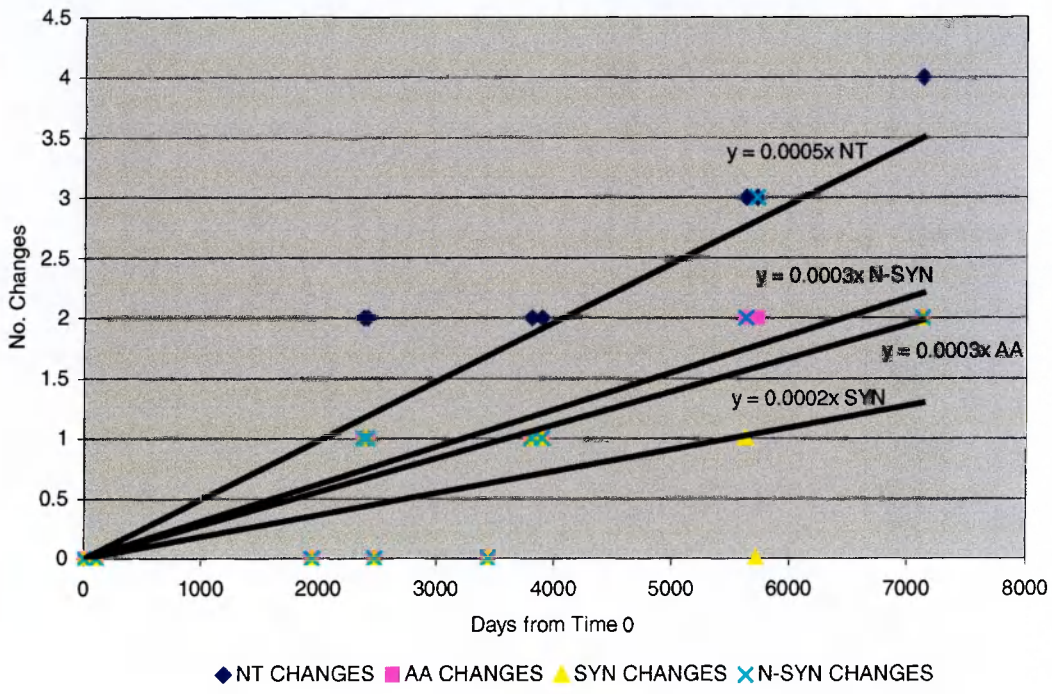
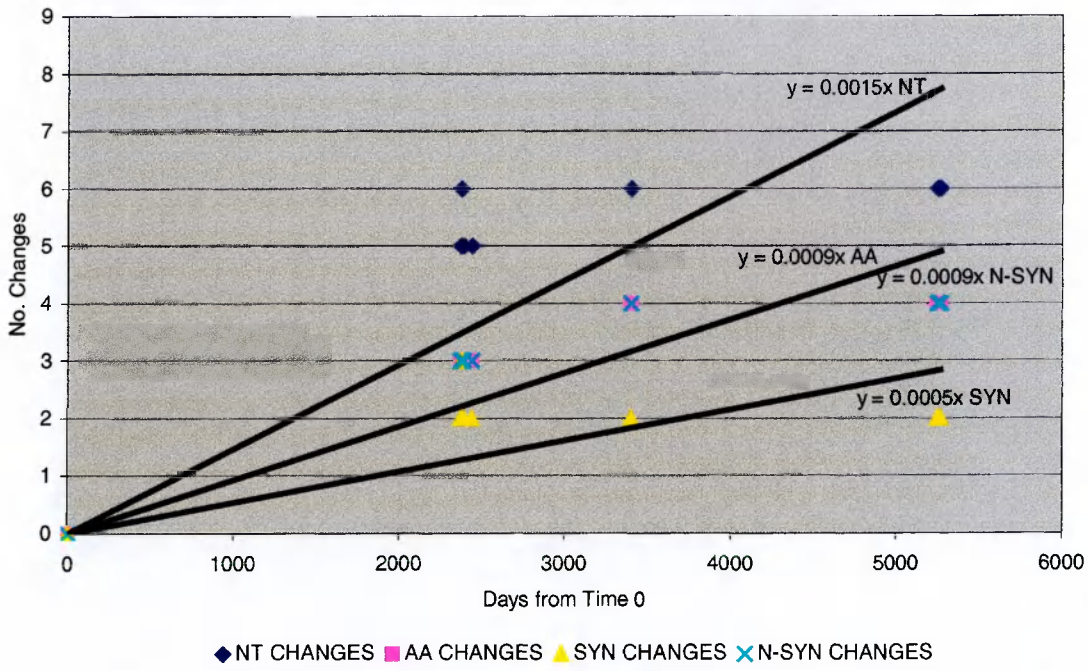


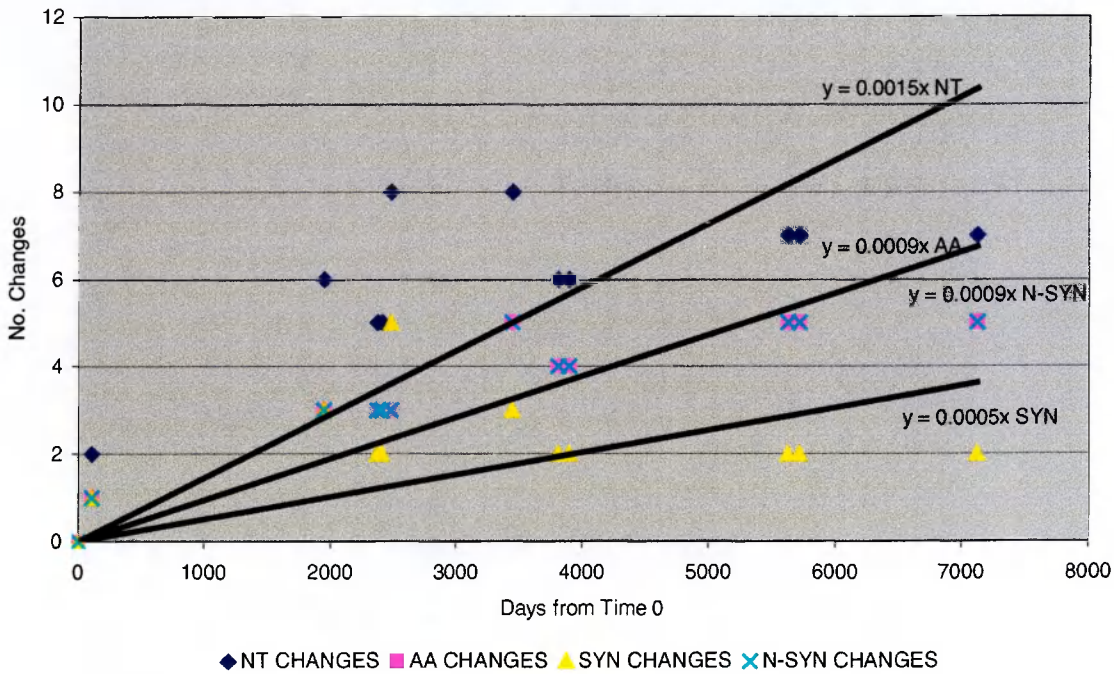
APPENDIX 14 Rate of change of M2 transmembrane domain from sensitive viruses (H1N1)



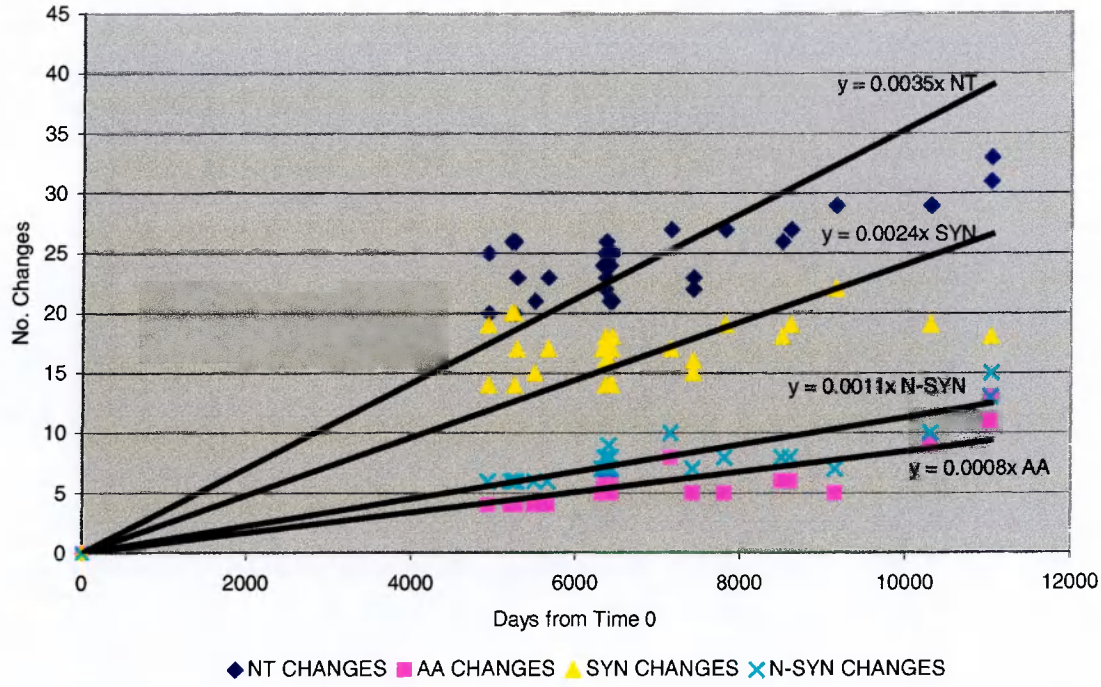
APPENDIX 15 Rate of change of M2 cytoplasmic domain from resistant viruses (H1N1)



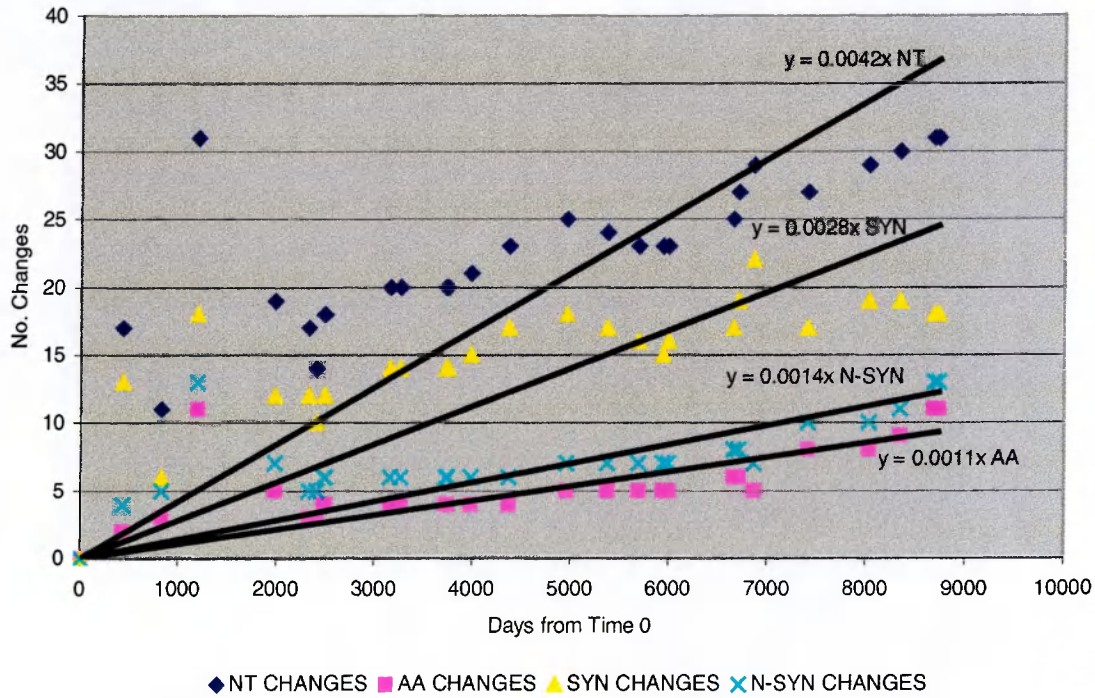
APPENDIX 16 Rate of change of M2 cytoplasmic domain from sensitive viruses (H1N1)



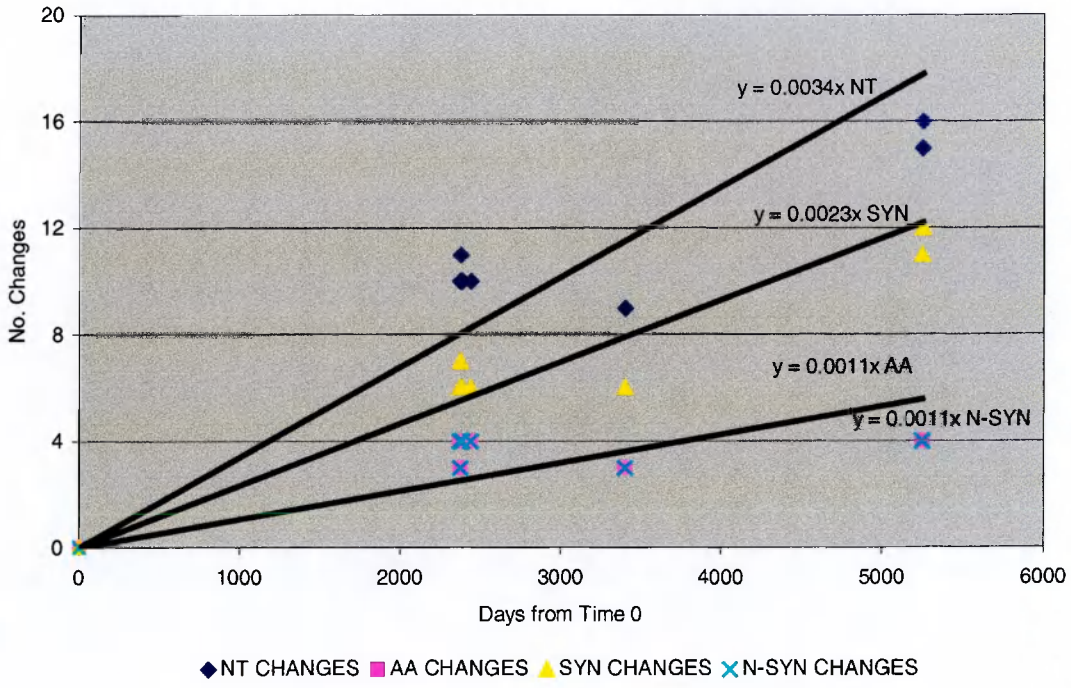
APPENDIX 17 Rate of change of M1 of resistant viruses (H3N2)



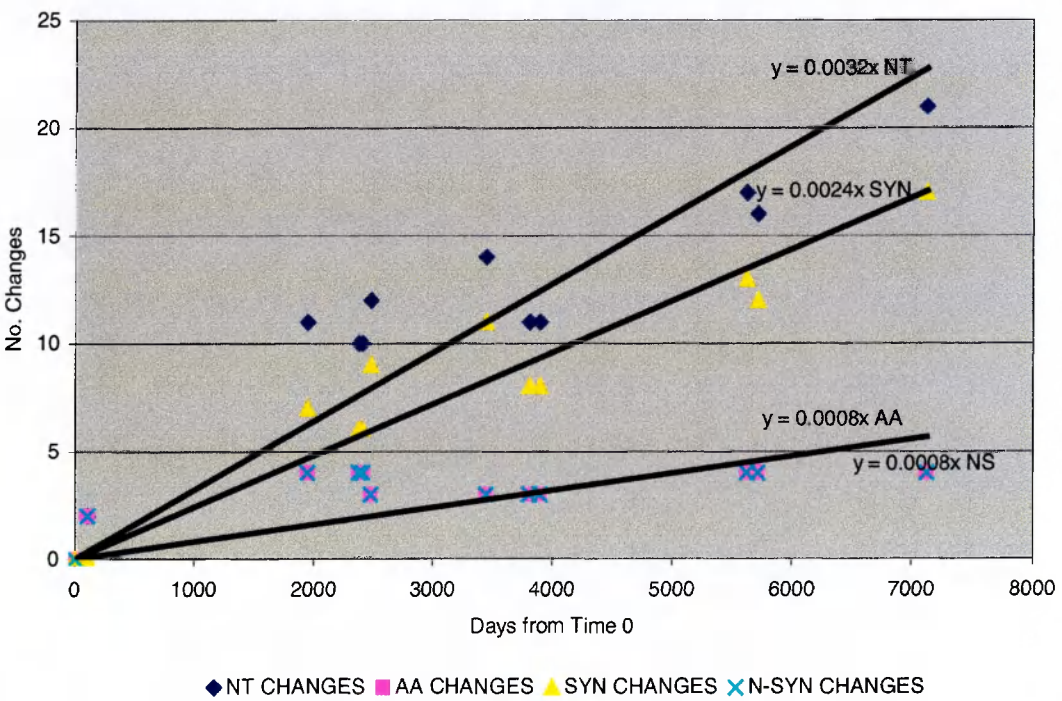
APPENDIX 18 Rate of change of M1 of sensitive viruses (H3N2)



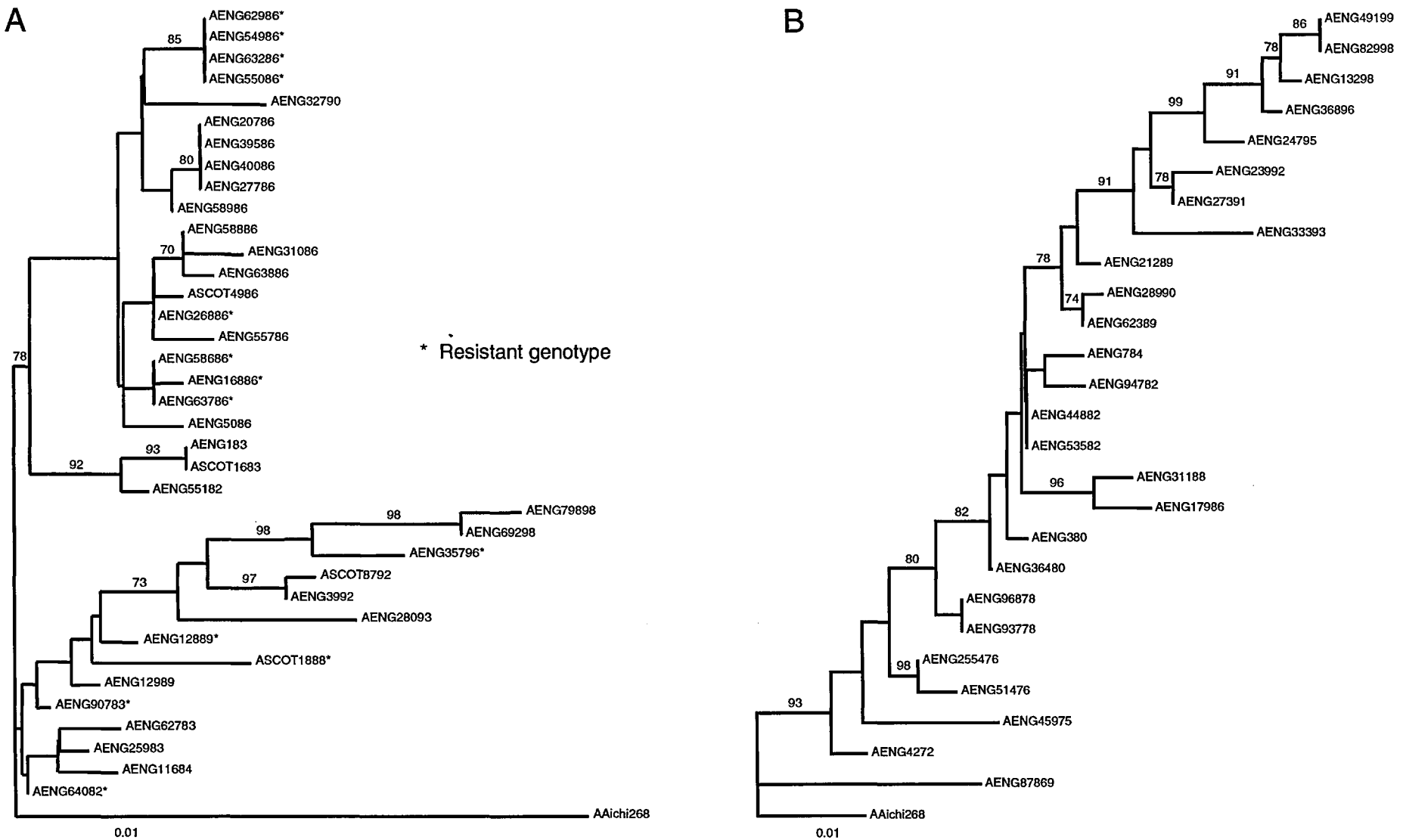
APPENDIX 19 Rate of change of M1 of resistant viruses (H1N1)



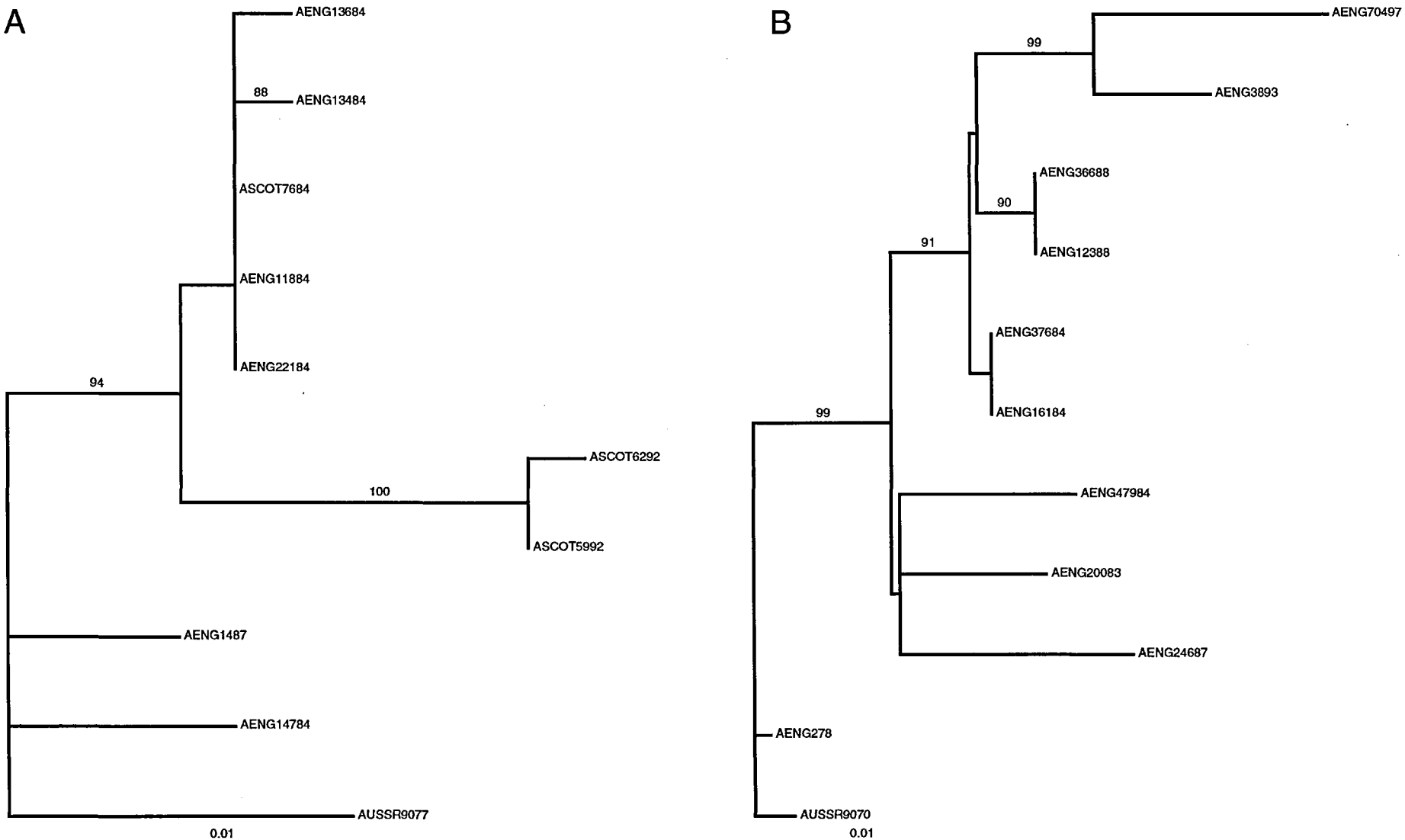
APPENDIX 20 Rate of change of M1 of sensitive viruses (H1N1)



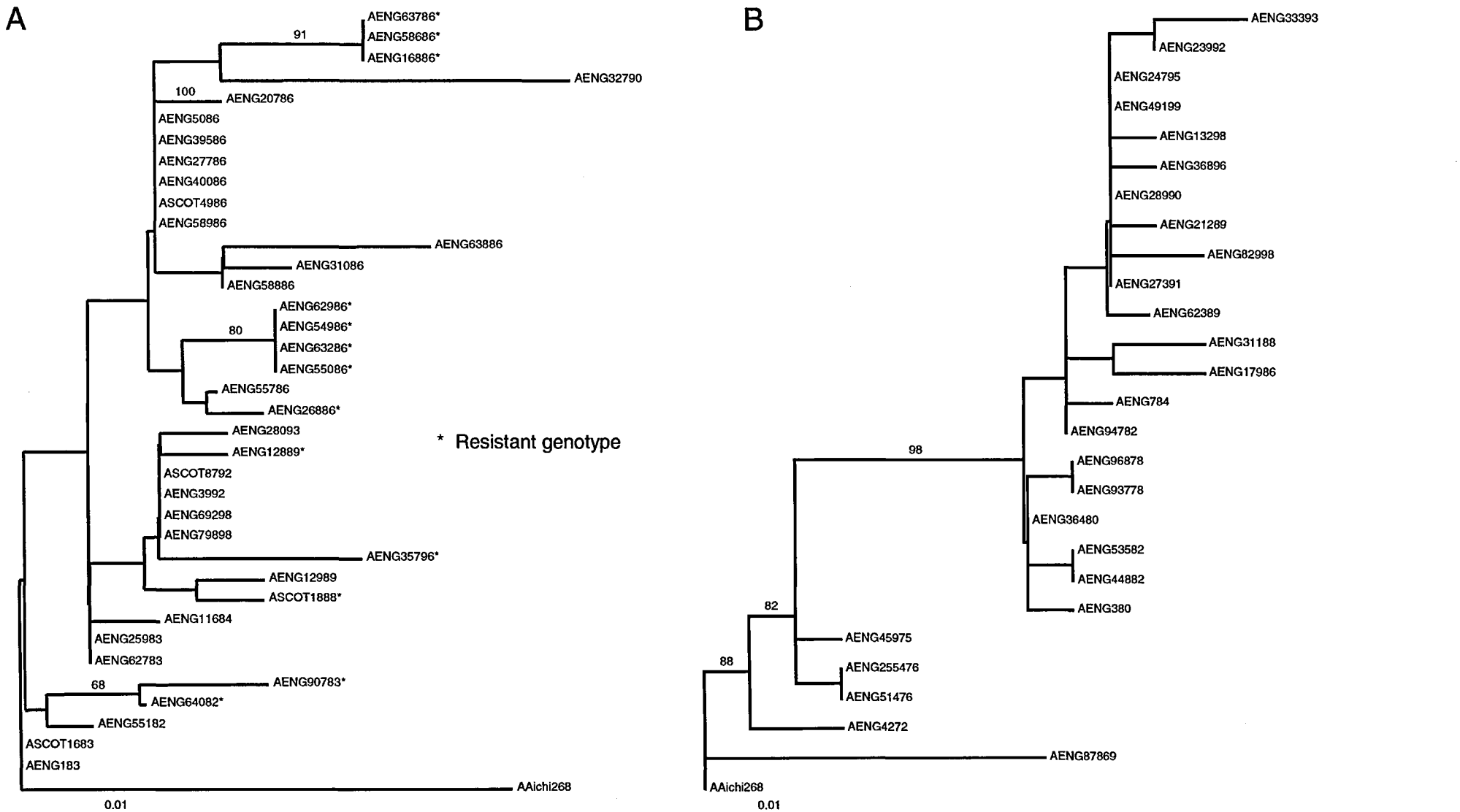
APPENDIX 21 Phylogenetic analysis of M1 nucleotide sequence originating from (A) resistant and (B) sensitive H3N2 viruses



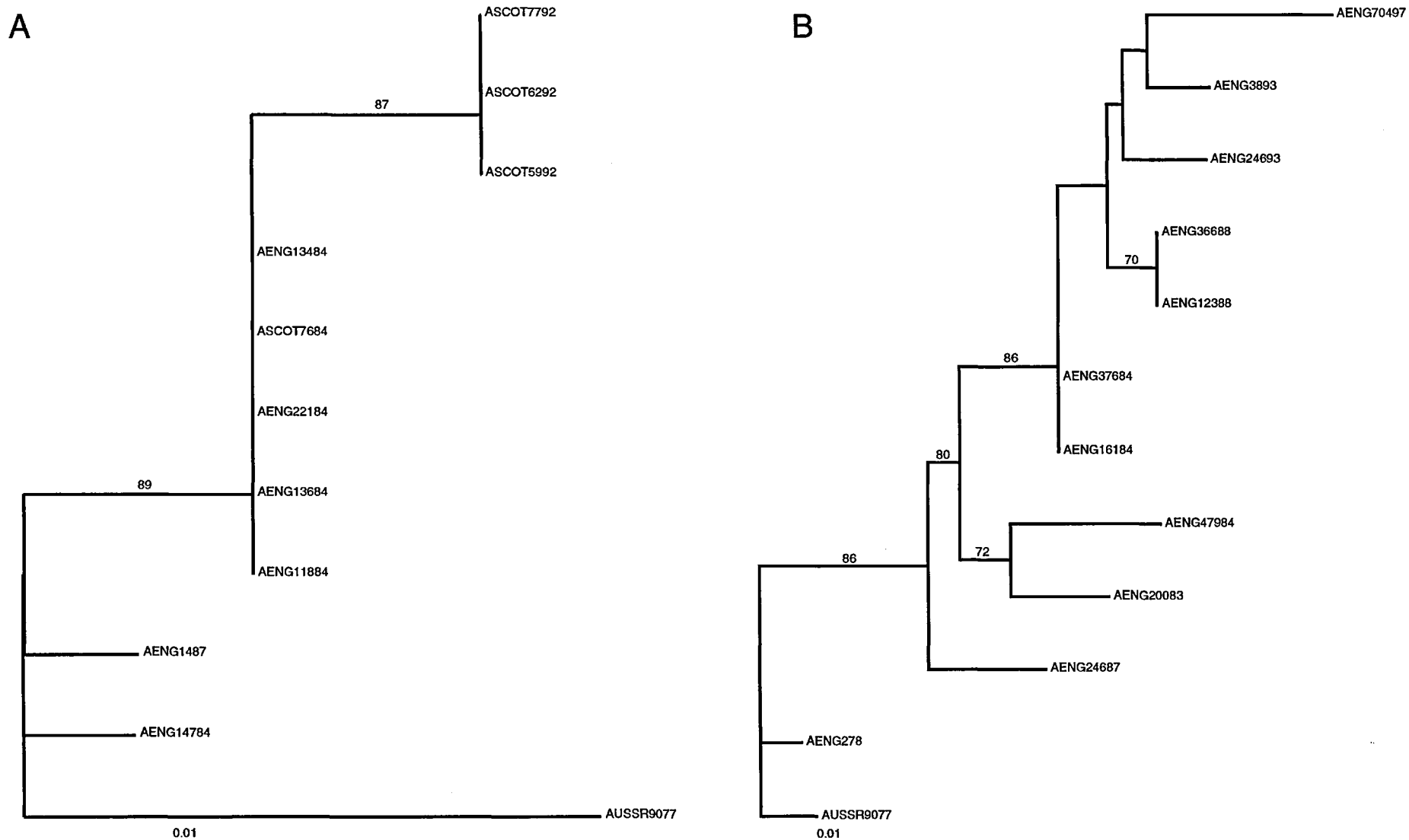
APPENDIX 22 Phylogenetic analysis of M1 nucleotide sequence originating from (A) resistant and (B) sensitive H1N1 viruses



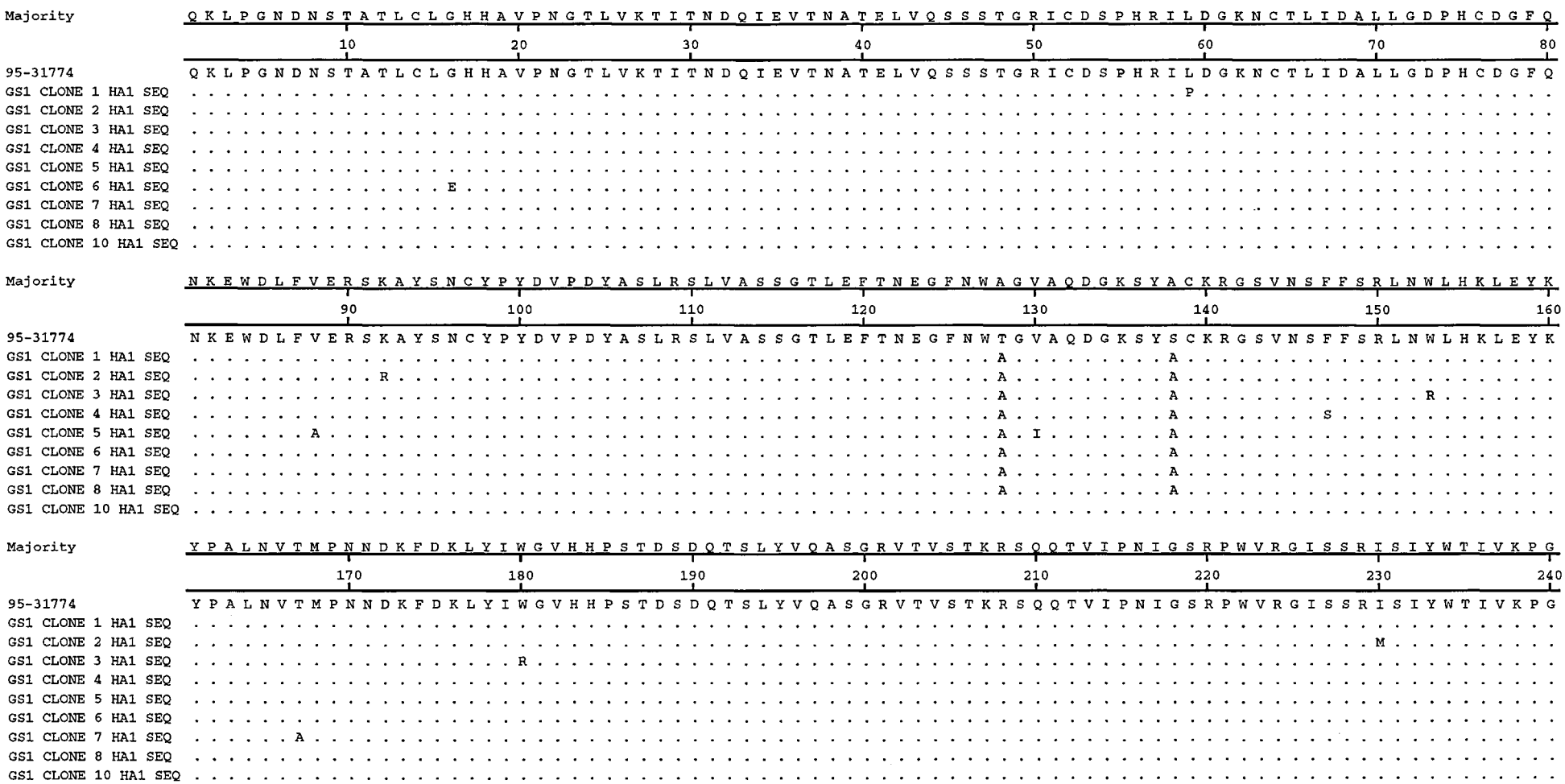
APPENDIX 23 Phylogenetic analysis of M2 nucleotide sequence originating from (A) resistant and (B) sensitive H3N2 viruses



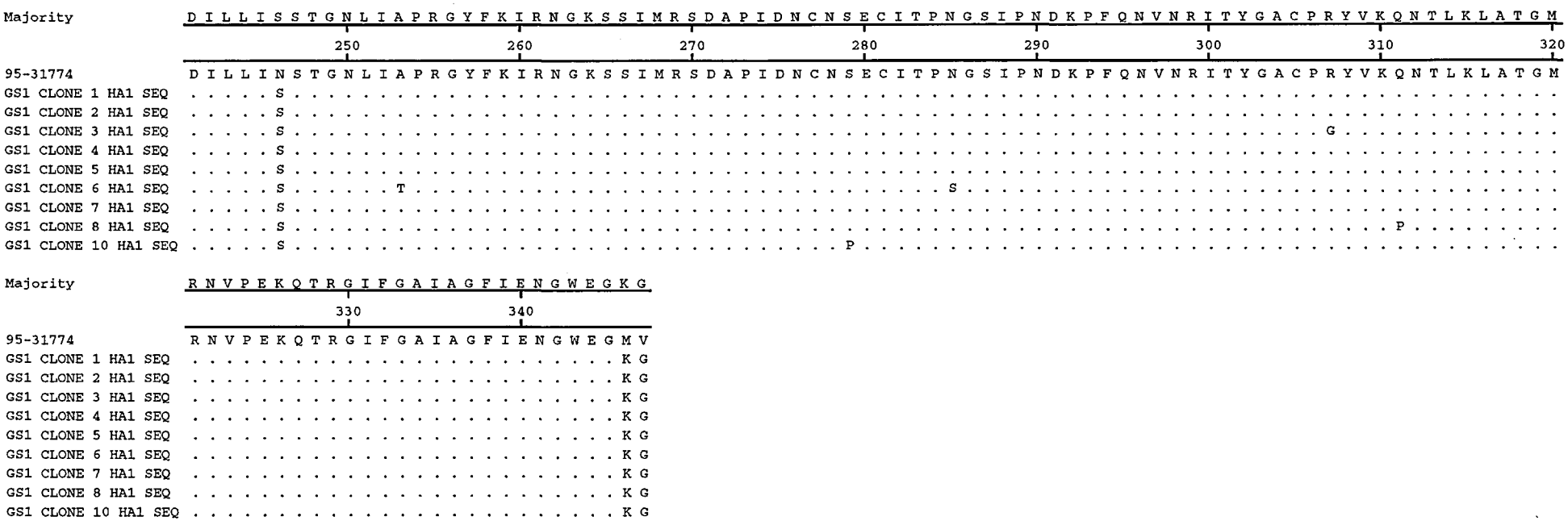
APPENDIX 24 Phylogenetic analysis of M2 nucleotide sequence originating from (A) resistant and (B) sensitive H1N1 viruses



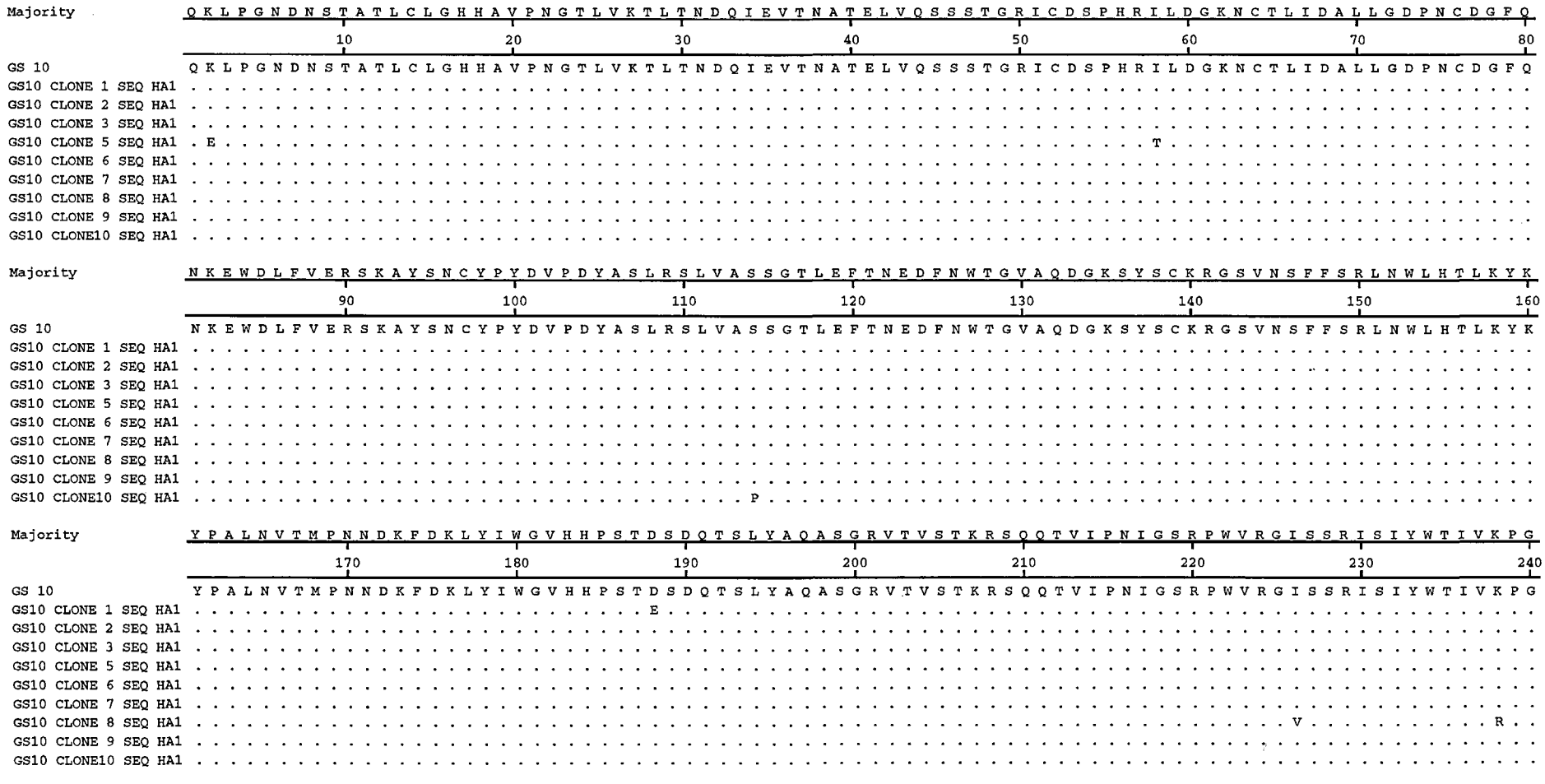
APPENDIX 25 Amino acid alignment of HA1 from GS1 clones



APPENDIX 25 Amino acid alignment of HA1 from GS1 clones (continued)



APPENDIX 26 Amino acid alignment of HA1 from GS10 clones



APPENDIX 26 Amino acid alignment of HA1 from GS10 clones (continued)

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Majority                D I L L I N S T G N L I A P R G Y F K I R N G K S S I M R S D A P I D N C N S E C I T P N G S I P N D K P F Q N V N R I T Y G A C P R Y V K Q N T L K L A T G M
                                    250                 260                 270                 280                 290                 300                 310                 320
GS 10                   D I L L I N S T G N L I A P R G Y F K I R N G K S S I M R S D A P I D N C N S E C I T P N G S I P N D K P F Q N V N R I T Y G A C P R Y V K Q N T L K L A T G M
GS10 CLONE 1 SEQ HA1    . V . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . S . . . . . . . . . . . . . . . .
GS10 CLONE 2 SEQ HA1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
GS10 CLONE 3 SEQ HA1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
GS10 CLONE 5 SEQ HA1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
GS10 CLONE 6 SEQ HA1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
GS10 CLONE 7 SEQ HA1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
GS10 CLONE 8 SEQ HA1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
GS10 CLONE 9 SEQ HA1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
GS10 CLONE10 SEQ HA1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

Majority                R N V P E K Q T R G I F G A I A G F I E N G W E G K G
                                    330                 340
GS 10                   R N V P E K Q T R G I F G A I A G F I E N G W E G L V
GS10 CLONE 1 SEQ HA1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
GS10 CLONE 2 SEQ HA1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
GS10 CLONE 3 SEQ HA1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
GS10 CLONE 5 SEQ HA1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
GS10 CLONE 6 SEQ HA1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
GS10 CLONE 7 SEQ HA1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
GS10 CLONE 8 SEQ HA1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
GS10 CLONE 9 SEQ HA1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
GS10 CLONE10 SEQ HA1    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
```